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## INTRODUCTION

Breast cancer is the most common malignancy observed among women in the Western hemisphere. It is estimated that one out of every nine women in the United States will develop the disease, underscoring an urgent need to develop novel therapeutic agents. Many lines of evidence support the notion that breast cancers develop in part due to the loss of functional tumor suppressors, and that the introduction of a functional tumor suppressor gene can inhibit the proliferation of breast cancer cells. Identification of such genes can lead to potential methods of treatment, including targeted gene therapy. We have developed a novel approach, called SETGAP (selectable expression of transient growth-arrest phenotype)(Pestov and Lau, 1994; Pestov et al., 1998), to isolate cDNAs of genes from normal human breast cells that can suppress the growth of breast cancer cells. This approach allows the identification of growth suppressors of breast cancer cells using a direct functional assay, and no specific information of the genes to be isolated is necessary. This novel approach promises to open new doors towards understanding the molecular player that regulate breast cancer cell growth and towards potential gene therapy strategies.

## BODY

### **Adaptation of the SETGAP procedure for selection of a cDNA library (Pestov et al., 1998).**

The original SETGAP procedure was designed to isolate potential growth inhibitory cDNA sequences from a small pool of target sequences (Pestov and Lau, 1994). We have adapted this procedure for the global selection of growth inhibitory sequences from a cDNA library (Pestov et al., 1998). This adapted procedure was shown to be efficacious. A novel cDNA sequence, Bop1Δ, was isolated.

**Construction of pEpiLac vectors (Li and Lau, 2000).** One key component of this selection is the IPTG-inducible expression vector pX11, which contains 15 copies of *lac* repressor binding sites in front of the MMTV basal promoter. pX11 was further modified to generate pX12 through the mutation of Oct1 sites in the MMTV promoter; this modification decreased the basal level expression of the luciferase reporter gene (Pestov and Lau, unpublished data). To facilitate the selection procedure further, we have created episomal expression vectors based on the fact that the presence of EBV *oriP* region and EBNA-1 gene on the same DNA molecule allows for extra-chromosomal replication in primate cells (Yates et al., 1985). These episomal vectors have several advantages over other vectors, including high efficiency of stable transfection, high level expression of transfected genes, easy recovery of transfected sequences, and the low complexity of transfected sequences when a population of DNA sequences is introduced (Deiss et al., 1995). These advantages make significant improvements upon the SETGAP procedure, making it easier to pinpoint the growth inhibitory cDNAs from cells survived SETGAP selection because of the low complexity of transfected sequences. To pursue all these advantages, pEpiLac vectors were constructed as IPTG-inducible episomal expression vectors with different multiple cloning sites (Li and Lau, 2000).

**Establishment of the MCF7/LAP5 cell line (Li and Lau, 2000).** LAP267 is a mammalian transcriptional activator which was derived by inserting VP16 transactivation domain after amino acid 267 of *lacI* repressor, and it also contains the SV40 nuclear localization signal (NLS) (Pestov and Lau, 1994; Baim et al., 1991). The transactivation activity of LAP267 was shown to be dependent on the presence of IPTG inducer (Pestov and Lau, 1994; Baim et al., 1991). In order to establish a cell line from the human breast cancer MCF7 to express LAP267 transactivator, MCF7 cells were cotransfected with pX6LAP267, which expresses LAP267 proteins, and pWLneo, which consistently expresses the neomycin-resistant gene. After G418 selection, 15 different clonal lines were analyzed. One clonal line, MCF7/LAP5 (called LAP5 hereafter), showed high level of IPTG-induced LAP267 transactivation activity, and this level is also dependent on the concentration of IPTG inducer. Thus, this cell line is appropriate for carrying out the SETGAP selection. Since we have adapted the use of episomal expression vectors, the ease of plasmid recovery using Hirt's extraction method. Episomal DNAs could be conveniently isolated from a stable population of LAP5 cells transfected with pEpiLac1p27 using Hirt's extraction method. After amplification in bacterial cells, the integrity of these DNAs were confirmed by restriction enzyme digestion and PCR analysis. Expression of transfected sequences (detected by a luciferase reporter) is inducible by nearly 300 fold using the episomal vector transfected into LAP5 cells (Li and Lau, 2000).

**Improved SETGAP procedure.** Since pEpiLac vectors efficiently express growth inhibitory genes in LAP5 cells and can be easily recovered using Hirt's extraction method, they are adapted into the SETGAP procedure in LAP5 cells. 1. A cDNA library is constructed by cloning cDNAs, in sense orientation, into the multiple cloning sites in pEpiLac vectors. 2. LAP5 cells are transfected with the library DNAs and selected against hygromycin. 3. Hygromycin selected cells are treated with IPTG to induce the expression of exogenous genes. 4. Proliferating cells are killed following BrdU/Hoechst dye/light selection, whereas, growth arrested cells will survive. 5. Growth arrested cells are rescued by the removal of IPTG inducer. 6. Episomal DNAs are extracted, amplified in bacteria and applied to the next round of selection. 7. After two rounds of selection, individual clones are applied to growth inhibition assays or DNA sequence analysis.

**Selection of growth inhibitory sequences from a human breast epithelial cell cDNA library.**

We have constructed a cDNA library in pEpiLac1 using mRNAs from normal human mammary gland cells using standard techniques. LAP5 cells were transfected with the library DNAs and about 6,000 hygromycin-resistant clones were obtained. These cells were then subjected to SETGAP selected. After one round of selection, extra-chromosomal DNAs were isolated from cells that survived the SETGAP selection and amplified in bacteria. The resulting DNA was applied to the second round of selection. Expression p27<sup>KIP1</sup>, a well-characterized growth inhibitor, gave rise to a similar number colonies that survived the SETGAP selection as the library DNA. By contrast, the parental LAP5 cells showed no such surviving cells following the selection. These results indicated that the pool of cDNAs from normal human epithelium contain sequences that can inhibit MCF7 cell growth.

Episomal DNA was harvested from the pool of LAP5 cells that survived the second round of SETGAP selection, and re-transfected into LAP5 cells for another round of selection. After the third round of selection, cells that survived the selection were grown to form colonies and 36 individual cell clones were picked and episomal DNA from these cells harvested. DNA from each individually picked LAP5 cell colony was amplified in bacteria. The bacterial transformation also served to purify the harvested episomal DNA into a single DNA species. DNA from each representative bacterial transformant was harvested, and again transfected into LAP5 cells to determine whether these individual DNA species can confer growth inhibition. After testing the 36 individually isolated clones, we identified 12 individual sequences that exhibit a growth arrest phenotype in the SETGAP assay. These sequences were named TGIF (Tumor growth inhibitory fragment) 1-12.

**Sequence and analysis of TGIFs.** The cDNA sequences of TGIF 1-12 were isolated and subjected to DNA sequence analysis. The sequences obtained were compared to those in the GenBank database. Results are summarized in Table 1. Some of these cDNAs correspond to known genes, whereas others correspond to novel genes or uncharacterized EST sequences. It is interesting to note that many of these sequences represent partial cDNAs rather than full-length cDNAs. This suggests that the partial cDNAs may encode dominant negative variants of normally expressed genes and interfere with the function of the full-length proteins. It is possible that these dominant negative variants may cause a more powerful growth arrest phenotype than other gene products that might be growth inhibitory; this may explain why in a genetic selection, the predominant products were partial cDNAs.

Among the 12 TGIF sequences isolated thus far, TGIF1 appeared promising since its expression confers a powerful growth inhibitory effect when expressed. Moreover, TGIF1 represents a novel gene sequence and characterization of this gene may lead to interesting new insights into the roles it may play in growth control. The TGIF1 clone was completely sequenced and compared to sequences in GenBank. TGIF1 corresponds a fragment of an uncharacterized gene with no known function (sequence KIAA0692; Ishikawa et al., 1998). The full-length 4.5 kb TGIF1 cDNA was also isolated from a human placental cDNA library and the sequence determined. The predicted open reading frame is shown in Fig. 1. Analysis of the encoded protein sequence did not yield any significant sequence homology to known proteins, and no functional domain that might indicate activity has been identified.

**Analysis of Bop1 (Strezoska et al., 2000).** Another sequence that came out of the SETGAP screen was Bop1 (Pestov et al., 1998). Bop1 is a novel mouse protein that contains WD40 repeats and is highly conserved through evolution. *bop1* is ubiquitously expressed in all mouse tissues examined and is upregulated during mid-G1 in serum-stimulated fibroblasts. Immunofluorescence analysis shows that Bop1 is predominantly localized to the nucleolus. In sucrose density gradients, Bop1 from nuclear extracts co-sediments with the 50-80S ribonucleoprotein particles that contain the 32S rRNA precursor. RNase A treatment disrupts these particles and releases Bop1 into a low molecular weight fraction. A mutant form of Bop1, Bop1 $\Delta$ , which lacks 231 amino acids in the N-terminus, is colocalized with wild type Bop1 in the nucleolus and in ribonucleoprotein complexes. Expression of Bop1 $\Delta$  leads to cell growth arrest in the G1 phase and results in a specific inhibition of the synthesis of the 28S and 5.8S rRNAs without affecting 18S rRNA formation. Pulse-chase analyses show that Bop1 $\Delta$  expression results in a partial inhibition in the conversion of the 36S to the 32S pre-rRNA, and a complete inhibition of the processing of the 32S pre-rRNA to form the mature 28S and 5.8S rRNAs. Concomitant with these defects in rRNA processing, expression of Bop1 $\Delta$  in mouse cells leads to a deficit in the cytosolic 60S ribosomal subunits. These studies thus identify Bop1 as a novel, non-ribosomal mammalian protein that plays a key role in the formation of the mature 28S and 5.8S rRNAs and in the biogenesis of the 60S ribosomal subunit.

## **KEY RESEARCH ACCOMPLISHMENTS**

- Construction of pEpiLac inducible episomal expression vectors
- Establishment of the MCF7/LAP5 cell line, which supports high levels of inducible expression
- Construction of a breast epithelial cell cDNA library in the pEpiLac expression vector
- Genetic selection yielded at least 12 cDNAs that inhibited cell growth, called TGIFs
- Sequence analysis of TGIFs revealed a number cDNA encoding proteins of unknown function
- Analysis of one of cDNA lead to the discovery of Bop1, a novel nucleolar protein involved in ribosomal RNA processing and ribosome assembly



## REPORTABLE OUTCOMES

### Publications:

1. Pestov, D.G., Grzeszkiewicz, T.M., Lau, L.F. (1998) Isolation of growth suppressors from a cDNA expression library. *Oncogene* 17: 3187-3197.
2. Li, Y. and Lau, L.F. (2000) An IPTG-inducible episomal expression system for exogenous genes in primate cells. *Biotechniques* 28: 577-81.
3. Strezoska, Z., Pestov, D.G., and Lau, L.F. (2000) Bop1 is a mouse WD40 repeat nucleolar protein involved in 28S and 5. 8S RRNA processing and 60S ribosome biogenesis. *Mol. Cell. Biol.* 20: 5516-28.

### Development of cell lines and reagents:

4. Development of the pEpiLac vector and the MCF7/LAP5 cell line, which allows highly inducible expression of foreign genes in MCF7 breast cancer cells with low background expression.
5. Isolation and characterization of a novel gene, *Bop1*, the sequence of which has been deposited into GenBank.

### Training:

This grant has allowed the continued training of a Postdoctoral fellow, Dr. Dimitri G. Pestov.

## CONCLUSIONS

A genetic system was established for isolating candidate tumor suppressor genes based on their growth inhibition phenotype. The IPTG-inducible episomal vectors, pEpiLac, were constructed. These vectors combined the advantages of inducible expression systems and extrachromosomal replication systems. The LAP5 cell line was derived from human breast cancer MCF7 cells by the introduction of the LAP267 transactivator. pEpiLac vectors expressed exogenous genes more efficiently than non-episomal vectors. Taking advantages of pEpiLac vectors and LAP5 cells, the SETGAP protocol is improved to make it easier to recover and identify exogenous growth arresting sequences. In addition, we have established an excellent inducible expression system tailored for the MCF7 breast cancer cells.

Using this novel system of selection for growth inhibitors, we have isolated and sequenced 12 growth inhibitory sequences. Many of these sequences represent fragments of known genes, while others represent previously unknown genes. It is noteworthy that the TGIFs isolated thus far are fragments of the full length cDNAs, suggesting that their encoded gene products are acting as dominant negative variants of the normally expressed full-length protein products. It is possible that these dominant negative variants comprise the more potent growth inhibitors when compared to normally expressed growth inhibitors, and therefore they are more likely to be isolated in a selection procedure designed to identify growth inhibitors. Inasmuch as we have already made sure that our cDNA library is comprised of mostly full-length cDNAs, the SETGAP procedure itself most likely exerts the selective forces in determining whether full-length cDNAs or partial cDNAs might be preferentially selected.

From this effort we have characterized a novel gene, *Bop1*, which encodes a previously unknown nucleolar protein that is involved in ribosomal RNA processing and ribosome assembly. We now have preliminary evidence that Bop1 also effects the cell cycle in a protein synthesis-independent manner. Thus, it is likely that Bop1 may play a role in mediating the cross-talk between ribosome biogenesis and cell cycle progression. Although a link between ribosome biogenesis and cell proliferation has been noted for many years, the mechanism of this linkage is unknown. The discovery of Bop1 identifies a molecular player in this cross-talk and further studies will likely reveal novel mechanisms of cell growth control and may identify new targets for cancer therapy.

It is important to note that thus far only a relatively small fraction of the complete library has been put through the SETGAP selection (6000 hygromycin resistant clones). Further selections exercises to examine a larger fraction of the library will likely yield new results. This study has provided the test of principle that the SETGAP procedure can work as a positive selection for growth inhibitory cDNAs, and further work is likely to identify new growth inhibitory mechanisms.

MIKGSRFKAF	STREDAEKFA	RGICDYFPSP	SKTSLPLSPV	KTAPLFSNDR	LKDGLCLSES	60
ETVNERANS	YKNPRTQDLT	AKLRKAVEKG	EEDTFSDLIW	SNPRYLIGSG	DNPTIVQEGC	120
RYNMHVAAK	ENQASICQLT	LDVLENPDFM	RLMYPDDDEA	MLQKRIRYVV	DLYLNTDPDKM	180
GYDTPLHFAC	KFGNADVNV	LSSHHLIVKN	SRNKYDKTPE	DVICERSKNK	SVELKERIRE	240
YLKGHYVPL	LRAEETSSPV	IGELWSPDQT	AEASHVSRYG	GSPRDPVLTL	RAFAGPLSPA	300
KAEDFRKLWK	TPPREKAGFL	HHVKKSDPER	GFERVGRELA	HELGYPWVEY	WEFLGCFVDL	360
SSQEGLRLE	EYLTQQEIGK	KAQQETGERE	ASCRDKATTS	GSNSISVRAF	<u>LDEDDMSLEE</u>	420
IKNRQNAARN	NSPPTVGAFG	HTRCSAFPLE	<u>Q</u> EADLIEAAE	PGGPHSSRNG	LCHPLNHSRT	480
LAGKRPKAPH	<u>G</u> EEAHLPPVS	DLTVEFDKLN	LQNIGRSVSK	TPDESTKTKD	QILTSRINAV	540
ERDLLEPSA	DQLGNHRRT	ESEMSARIAK	MSLSPSSPRH	EDQLEVTREP	ARRLFLFGEE	600
PSKLDQDVLA	ALECADVDPH	QFPAVHRWKS	AVLCYSPSDR	QSWPSPAVKG	RFKSQLPDLS	660
<u>G</u> PHSYSPGRN	SVAGSNPAKP	GLGSPGRYSP	VHGSQLRRMA	RLAELAAL*		

Note: = marks the start codon in TGIF1

E450 is K in TGIF1;

H490 is R in TGIF1;

G661 is A in TGIF1

**Figure 1. Deduced protein sequence encoded by TGIF1.** The deduced protein sequence encoded by the full-length cDNA corresponding to TGIF1 is shown. When compared to the KIAA0692 sequence (Ishikawa et al., 1998), the TGIF sequence showed differences in three amino acids (underlined).

clone number	Description
<b>TGIF1</b>	<b>1.4 kb fragment of uncharacterized sequence KIAA0692 (Ishikawa K. et al. 1998. Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res. 5(3):169-176.)</b>
<b>TGIF2</b>	<b>1.2 kb sequence. About 100 bp was sequenced at 5', which is about 97% identical to 695-876 interval of the human ras-related krev-1 gene (1579 kb long).</b>
<b>TGIF3</b>	<b>0.6 kb sequence, partial DNA sequencing showed similarity to the ribosomal protein L11 gene</b>
<b>TGIF4</b>	<b>0.7 kb sequence, partial DNA sequencing showed similarity to the human eIF4D and eIF5A</b>
<b>TGIF5</b>	<b>1 kb sequence, partial DNA sequencing showed similarity to HGR74 cDNA</b>
<b>TGIF6</b>	<b>1.7 kb sequence, partial DNA sequencing showed similarity to the Ca<sup>++</sup> and phospholipid-binding protein synaptotagmin 4.</b>
<b>TGIF7</b>	<b>0.9 kb unidentified sequence</b>
<b>TGIF8</b>	<b>0.7 kb fragment of a novel gene which gave a transcript of about 2 kb as determined in Northern blot analysis.</b>
<b>TGIF9</b>	<b>0.7 kb sequence similar to human chaperonin 10 cDNA</b>
<b>TGIF10</b>	<b>250 bp fragment similar to ZNF195 Kruppel zinc finger protein.</b>
<b>TGIF11</b>	<b>0.7 kb sequence, partial DNA sequencing showed similarity to human metallothionein-II cDNA</b>
<b>TGIF12</b>	<b>0.7 kb sequence, partial DNA sequencing showed similarity to the abundant gene for human ribosomal protein L21.</b>

**Table 1. Characterization of TGIF sequences isolated through SETGAP.** The 12 TGIF sequences isolated through SETGAP selection in LAP5 cells were isolated and sequenced. The sizes of the TGIF sequences are shown, as are their sequence characterization.

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## FINAL REPORTS

### Publications:

1. Pestov, D.G., Grzeszkiewicz, T.M., Lau, L.F. (1998) Isolation of growth suppressors from a cDNA expression library. *Oncogene* 17: 3187-3197.
2. Li, Y. and Lau, L.F. (2000) An IPTG-inducible episomal expression system for exogenous genes in primate cells. *Biotechniques* 28: 577-81.
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### Personnel receiving pay from the research effort:

Dimitri G. Pestov, Ph.D. (1997-2000)



# Isolation of growth suppressors from a cDNA expression library

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We describe an experimental procedure for the isolation of growth inhibitory sequences from a complex cDNA library. This approach first takes advantage of the SETGAP technique (selectable expression of transient growth arrest phenotype) to enrich for growth inhibitory sequences, followed by a screening procedure to identify individual cDNAs that inhibit cell proliferation. Here we provide a detailed description of the experimental protocol and report the characterization of two cDNA sequences isolated in our initial screen of a mouse cDNA library. One of these cDNAs encodes the mouse ubiquitin-conjugation enzyme UbcM2. The other encodes a truncated form of a novel WD40 repeat protein, named Bop1, which is conserved from yeast to human. Together, these results demonstrate a new approach for the isolation of growth suppressors from cDNA libraries, and identify a previously unknown gene likely to be involved in growth control.

**Keywords:** inducible vector; Rb; 3T3 cells

## Introduction

Cell proliferation can be regulated both positively and negatively. Considerable progress has been made in understanding the mechanisms that lead to activation of the proliferative program. Recent studies have also demonstrated the existence of multiple negative control mechanisms, which can intervene at several checkpoints to prevent cell cycle progression if previous cycle events have not been properly completed (Hartwell and Weinert, 1989; Elledge, 1996; Paulovich *et al.*, 1997). The importance of these mechanisms is underscored by the fact that the loss of function of the proteins involved, such as p53, can lead to genetic damage and neoplasia (Donehower *et al.*, 1992; Hartwell and Kastan, 1994; Cross *et al.*, 1995; Harper and Elledge, 1996). During development, most cells switch from active proliferation to nonreplicative, differentiated state. Although growth-arrested cells constitute the majority of cells in the organism, the molecular mechanisms that establish and maintain their replicative arrest are only poorly understood.

Positive regulators of cell growth may confer a growth advantage on cells expressing them, or may indeed overpower the cellular growth control machinery and lead to uncontrolled proliferation. Consequently, a plethora of oncogenes have been identified by means of retroviral transduction and gene transfer.

In contrast, the identification of genes that can suppress proliferation in a dominant manner has proven more difficult. Cells expressing a gene that cause growth arrest may be eliminated from a growing culture, and these cells do not have a phenotype for which a selection can be easily applied. The use of genetic methods for studying growth-inhibitory pathways has therefore been limited.

We have recently developed a method that allows the isolation of growth-inhibitory DNA sequences by genetic selection in mammalian cells (Pestov and Lau, 1994). This method, called SETGAP (selectable expression of transient growth-arrest phenotype), utilizes a library that potentially contains growth-inhibitory sequences cloned in an inducible expression vector. After transfection into mammalian cells, expression of library DNA is transiently induced to block growth of those cells that have incorporated growth-inhibitory sequences. These cells are then isolated from the transfected population by selective killing of cells that are capable of DNA replication.

In the previous study, we tested the feasibility of SETGAP in the selection of short inhibitory fragments of a small group of known growth-related genes (Pestov and Lau, 1994). In an effort to devise a method to identify previously unknown growth-inhibitory genes, we have developed a two-step procedure based on SETGAP. Using this approach, we have isolated two cDNA clones that can reversibly arrest cell growth. One of these clones encodes a known gene, the mouse ubiquitin-conjugating enzyme UbcM2. The other encodes an amino-terminal truncated sequence of a novel gene with unknown function, *bop1*. Expression of both of these cDNAs in NIH3T3 fibroblasts results in a reversible inhibition of G1 progression. These results suggest that our experimental approach can be a useful strategy for identification of novel growth-inhibitory genes in cDNA libraries.

## Results

### *Genetic selection for the enrichment of growth inhibitory sequences*

We have developed a two step approach to isolate growth-inhibitory cDNA clones from a complex cDNA library. First, we applied the SETGAP selection procedure (Pestov and Lau, 1994) to enrich for sequences that are capable of arresting cell growth when expressed. Second, we subjected the enriched cDNA sequences to a screening procedure to identify individual growth-inhibitory clones.

As a source of mRNA for our cDNA library, we used primary mouse embryo fibroblasts cultured

according to the 3T3 regimen to the point of crisis, whereupon these cells lost their proliferative potential and took on morphological characteristics reminiscent of senescent cells (Todaro *et al.*, 1965; Todaro and Green, 1963). mRNA from these fibroblasts was used to prepare a cDNA library using the vector pX11, which allows IPTG-inducible expression in NIH3T3-derived LAP3 cells (Pestov and Lau, 1994). The cDNA library was transfected into LAP3 cells; approximately  $10^4$  stably transfected clones were divided into several pools and subjected to selection using a modified SETGAP procedure that was adapted for a complex full-length cDNA library. The essential steps in this selection are as follows (Figure 1): (1) Cells that incorporate BrdU while transfected sequences are expressed under IPTG induction are selectively killed; (2) Cells that are growth-inhibited due to expression of transfected sequences survive the selective killing and are rescued by the removal of IPTG. Another cycle of IPTG induction and BrdU/light treatment can be applied to the rescued cells to reduce background; (3) Transfected cDNA sequences are recovered by PCR and recloned into pX11. Since cells rescued after one round of selection may contain multiple sequences because of cotransfection, sequences recovered from them can be pooled into secondary libraries and subjected to another round of selection to further reduce complexity. After the second round, the rescued sequences can be analysed individually.

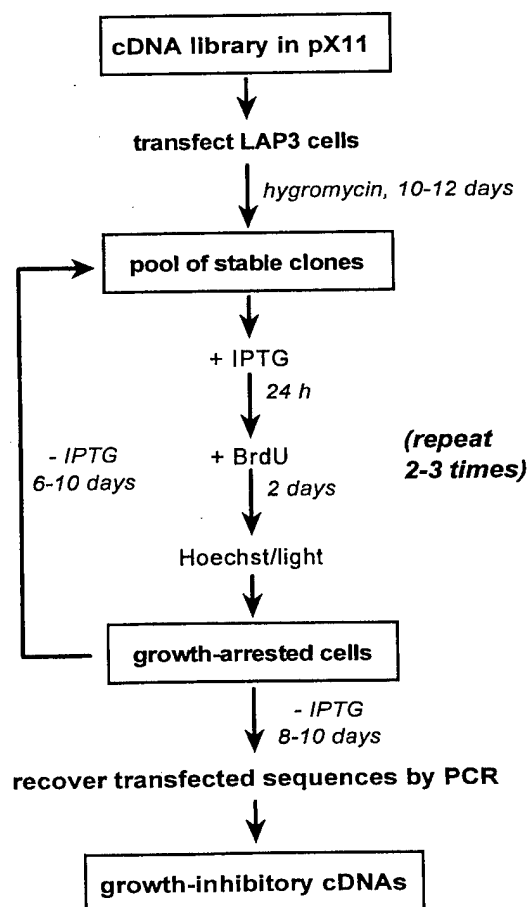


Figure 1 Schematic representation of the SETGAP procedure for selection of growth-inhibitory cDNA sequences

LAP3 cells transfected with the cDNA library were treated with IPTG to induce expression of the transfected sequences, which resulted in increased survival of cells after BrdU/light treatment (Figure 2a). A similar increase was observed when LAP3 cells were transfected with a growth-inhibitory variant of JunB (11-11) (Pestov and Lau, 1994), whereas cells transfected with negative controls (pX11 empty vector or pX8B6-luc) did not show a similar effect. Cells that survived one round of selection were expanded and transfected sequences were recovered by PCR, generating mixtures of products that ranged from 300 bp to >3 kb in length. The rescued sequences were subjected to another round of selection. To this end, PCR products >700–800 bp in length recovered from surviving cells from each dish were isolated from an agarose gel and recloned into pX11 to create a secondary library. Twenty secondary libraries thus obtained were transfected into LAP3 cells, generating a few thousand stable clones in each transfection. Four out of the 20 secondary libraries were positive in SETGAP selection, yielding surviving cells. The transfected sequences recovered by PCR from these cells were significantly less complex than cDNAs isolated after one round of selection. However, attempts to reduce this complexity further by additional rounds of selection were unsuccessful, most likely due to unavoidable cotransfection of multiple sequences into a single cell in the calcium phosphate-mediated transfection procedure.

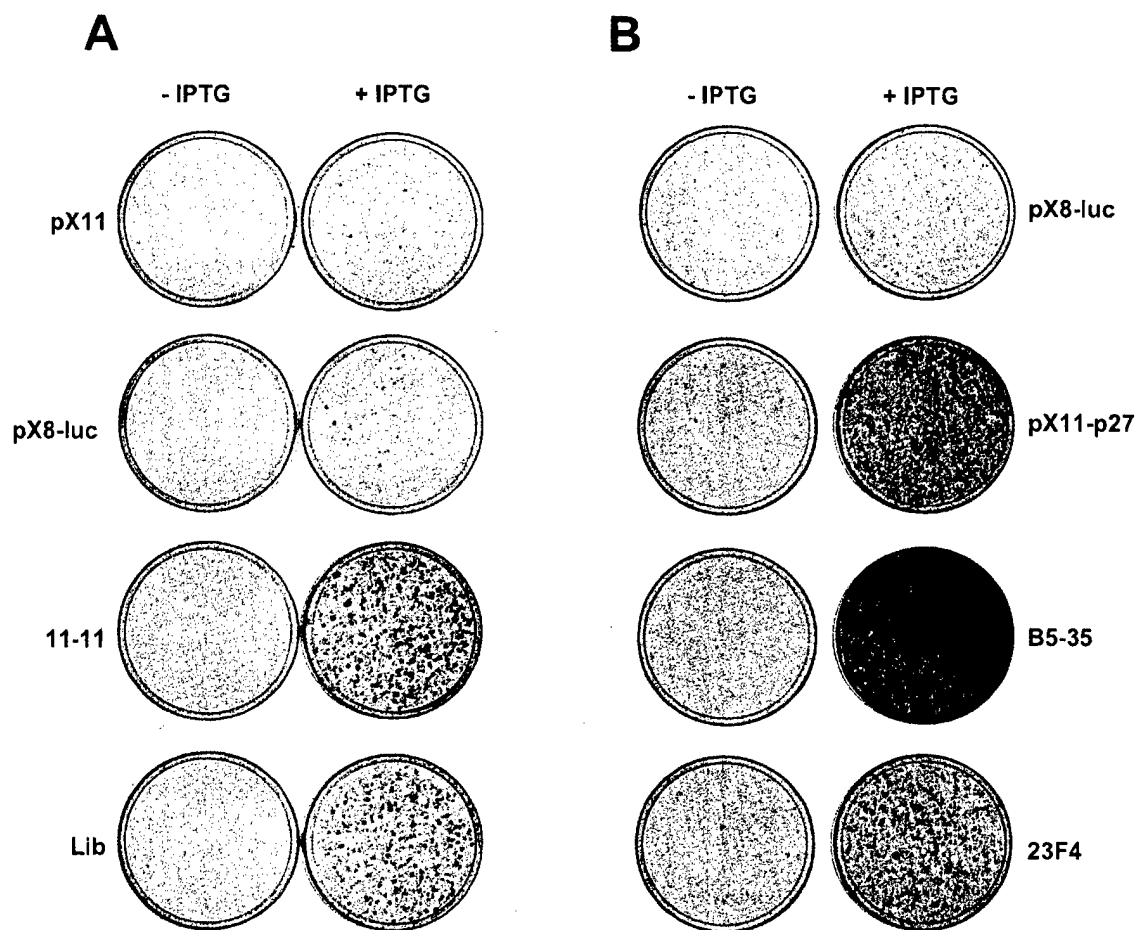
#### Screening for individual growth-inhibitory cDNA clones

Sequences recovered from each of the four cell populations that survived the second round of selection were cloned into pX11 and 30–50 individual plasmid clones were isolated at random. Plasmid DNA of each clone was digested with *Hinf*I to produce a distinct pattern to exclude identical clones from further analysis. In order to identify individual growth inhibitory sequences, we devised a screening procedure, SETGAP-2. Clones were cotransfected with a  $\beta$ -galactosidase marker plasmid into LAP3 cells, which were subjected to one cycle of IPTG induction and BrdU/light treatment and then stained with X-gal (technical details are provided in Materials and methods). The growth of blue colonies surviving the BrdU/light procedure indicated the growth-inhibitory potential of the transfected sequence. Using this screening method, we isolated two growth-inhibitory clones derived from two separate cell populations. The two isolated growth-inhibitory clones contained inserts of 1.2 kb (clone 23F4) and 1.75 kb (clone B5-35).

#### Expression of 23F4 or B5-35 inhibits cell proliferation

Expression of 23F4 or B5-35 in LAP3 cells led to a dramatic increase in the number of cells that survived the SETGAP procedure (Figure 2b). To confirm that this effect was due to growth inhibition, we pooled cells from approximately  $10^3$  stable clones derived by transfection with 23F4, B5-35 or control constructs pX11 and pX11-p27, and counted the number of cells after three days of culture in the presence or absence of IPTG (Figure 3). Pools of transfected cells rather than clonal cell lines were examined in this experiment to





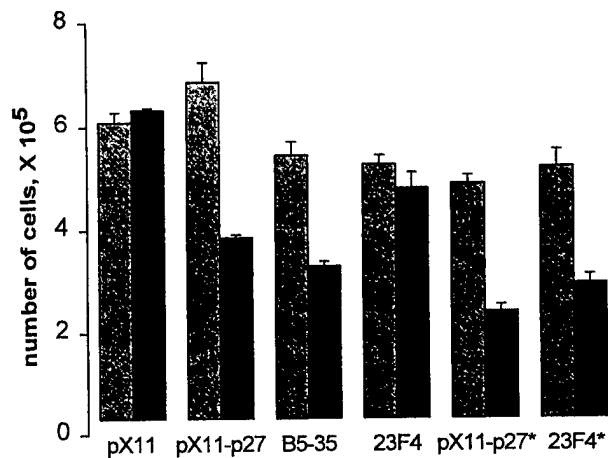
**Figure 2** SETGAP selection for growth inhibitory cDNAs. (a) Cells were transfected with either the cDNA library (Lib), the growth-inhibitory construct 11-11 (a truncated variant of JunB) (Pestov and Lau, 1994), or the negative control plasmids pX11 (empty vector) and pX8-luc (vector driving expression of the luciferase gene). Stable clones obtained from each transfection were pooled, and  $10^5$  cells from each pool were subjected to two cycles of BrdU/light treatment to kill cells that synthesize DNA. Cells were tested for survival of BrdU/light treatment either in the presence or absence of 1 mM IPTG to regulate expression of transfected sequences. After the second BrdU/light treatment, surviving cells were allowed to grow for 7 days in the absence of IPTG and then stained with crystal violet. (b) Cell survival after BrdU/light treatment upon induction of transfected sequences (+ IPTG). Cells were transfected with either the negative control pX8-luc, the positive control pX11-p27 (a p27Kip1 expressing plasmid), and clones B5-35 and 23F4. Experiments were carried out as in (a), except that cells were subjected to only one cycle of BrdU/light treatment before surviving cells were rescued

eliminate any clonal effects on growth rates. While the presence of IPTG did not significantly alter the growth of cells transfected with pX11, induction of B5-35 resulted in a significant decrease in the rate of cell proliferation, comparable to the effect of expression of the transfected cdk inhibitor p27Kip1 (Figure 3). Induction of expression of 23F4 in this assay resulted in a modest inhibition of the growth rate, which was consistent with a relatively smaller effect observed in SETGAP (see Figure 2b). When we first subjected the pool of cells transfected with 23F4 to one round of SETGAP selection and used the surviving cells for the cell proliferation assay, these pre-selected cells displayed a clear reduction in their growth rate upon IPTG treatment (Figure 3). The observed increase in the growth-inhibitory effect after SETGAP selection likely reflects the enrichment in the proportion of cells that express the 23F4-encoded sequence at a sufficiently high level to cause growth inhibition. Parallel selection of cells transfected with the inducible p27Kip1 construct also increased the observed growth-inhibitory effect of its expression (Figure 3). Taken together,

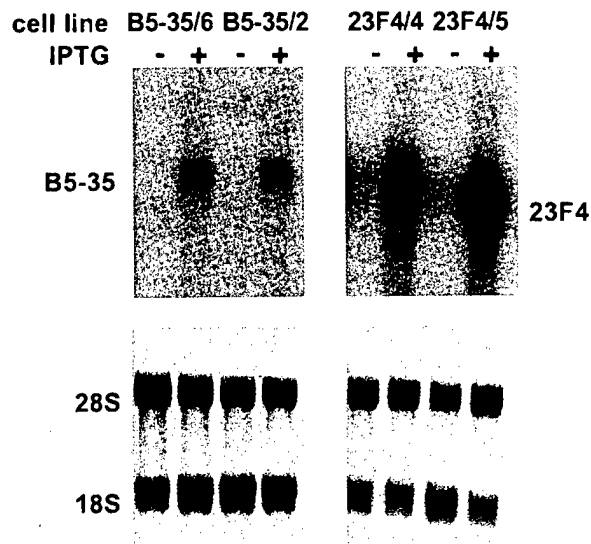
these results show that expression of B5-35 and 23F4, identified in a SETGAP assay, can indeed inhibit proliferation in LAP3 fibroblasts.

#### *Expression of B5-35 or 23F4 leads to G1 arrest*

To investigate the nature of the growth inhibition conferred by expression of cDNA sequences in B5-35 and 23F4, we cotransfected these plasmids with pHyg into LAP3 cells and generated stable clonal lines in which expression of the transfected sequences was inducible by IPTG (Figure 4). We tested the effect of expression of these sequences on the ability of cells synchronized by serum starvation to initiate DNA synthesis. Several independently obtained stable lines were serum-starved and then restimulated with 10% serum in the presence of BrdU. The number of cells entering S phase was assessed by immunohistochemical staining of BrdU-positive nuclei. Induction of B5-35 and 23F4 in several tested lines inhibited the ability of cells to enter S phase, whereas no effect was observed in the control cell line transfected with the empty



**Figure 3** Expression of B5-35 or 23F4 inhibits cell proliferation. Stable clones obtained by transfection of indicated plasmids into LAP3 cells were pooled and seeded into 35-mm dishes ( $3 \times 10^4$  cells per dish). Cells were counted after 3 days of growth, before cells reached saturation density, in medium with 1 mM IPTG (solid bars) or without IPTG (shaded bars). Error bars show variations among duplicate dishes. Two sets of bars (pX11-p27 and 23F4) labeled with an asterisk (\*) show data obtained with transfected cells pre-selected with one round of SETGAP



**Figure 4** Inducible expression of B5-35 and 23F4 mRNA in clonal cell lines. Individual clones of LAP3 cells stably transfected with B5-35 (lines B5-35/6 and B5-35/2) or 23F4 (lines 23F4/4 and 23F4/5) were either untreated (-) or treated (+) with 1 mM IPTG for 9 h. Total RNA (2.5  $\mu$ g for left panel and 1.5  $\mu$ g for right panel) was analysed by Northern blotting with the corresponding cDNA probes. Lower panel: equal loading in each lane was shown by methylene blue staining of the same blot to reveal ribosomal RNAs

vector (Figure 5). Expression of B5-35 (line B5-35/6) and 23F4 (line 23F4/4) blocked DNA synthesis in 80% of cells in this experiment; expression of 23F4 in another clonal line, 23F4/5, resulted in approximately 50% fewer cells undergoing DNA synthesis. In all stable cell lines examined, induction of either 23F4 or B5-35 was not apparently toxic and did not induce any noticeable increase in cell death even after a prolonged IPTG treatment.

To test whether the inhibition of S phase entry might be due to G1 arrest, we analysed the status of the retinoblastoma tumor suppressor protein (Rb), which is hyperphosphorylated prior to G1 exit, in inducible cell lines B5-35/6 and 23F4/4. These cells were synchronized by serum starvation and then stimulated with 10% serum. Induction of B5-35 with IPTG strongly inhibited Rb phosphorylation (Figure 6). Although Rb phosphorylation was not inhibited in cells expressing 23F4, it was considerably delayed (compare hypophosphorylated Rb at 12 h in 23F4-expressing and -non-expressing cells). These data indicate that the antiproliferative effects of both B5-35 and 23F4 are, at least in part, due to restriction of progression through G1 prior to Rb phosphorylation.

#### Clone 23F4 encodes a ubiquitin-conjugation enzyme

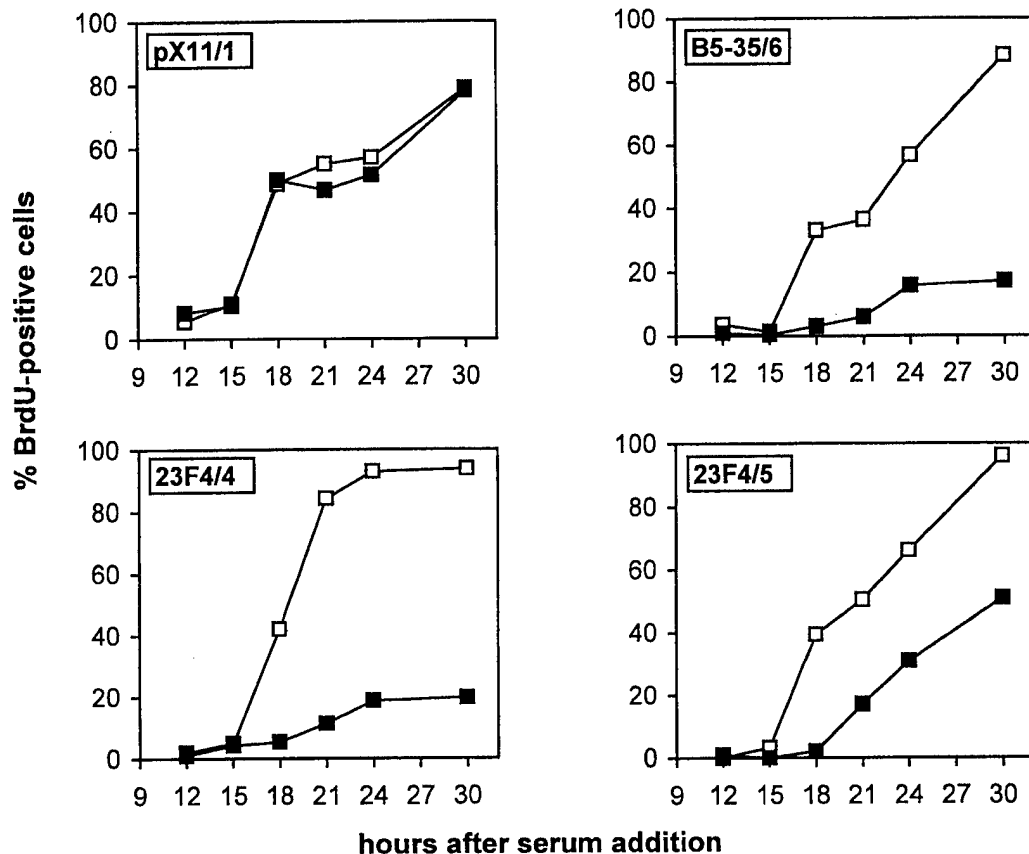
Sequence analysis revealed that clone 23F4 encodes the entire reading frame of the mouse ubiquitin-conjugating enzyme UbcM2. Compared to the previously published UbcM2 sequence (Genebank accession no. X92664), 23F4 (Genebank accession no. AF003346) differs by a single A-G substitution within the coding region (nucleotide 94 in 23F4) and contains a longer 3'-untranslated region. The nucleotide substitution was not due to a mutation that occurred during SETGAP selection, since it was also present in a sequence independently obtained from primary mouse fibroblasts by reverse transcription-PCR using UbcM2-specific primers.

To determine whether growth inhibition by de-regulated expression of UbcM2 is dependent on its enzymatic activity, we created a single amino acid substitution mutant construct in which the cysteine-145 residue at the active site of this enzyme (Matuschewski *et al.*, 1996) was replaced with a serine residue. This mutation abolished inhibition of cell growth as judged by the SETGAP assay, suggesting that it is the enzymatic function of UbcM2 that causes the growth inhibition (Figure 7), rather than any non-productive interactions that may result from overexpression of the protein.

#### Clone B5-35 identifies a novel WD40 repeat protein, Bop1

We isolated a full-length cDNA sequence corresponding to B5-35 from a mouse library as described in Materials and methods. The isolated cDNA, designated *Bop1* (block of proliferation), was 2476 bp long, in agreement with the size of a single transcript detected in mouse cells. The B5-35 clone (1737 bp) initiates at nt 747 of the *bop1* sequence, having deleted the 5' end (Figure 8). The B5-35 cDNA contains a single long open-reading frame that starts with two closely located ATG codons, the second of which (nt. 799; Figure 8) is in a good Kozak context. We constructed an expression vector encoding a deletion mutant, Bop1 $\Delta$ , whose translation is initiated with this codon. Expression of Bop1 $\Delta$  led to growth inhibitory effects indistinguishable from those of B5-35 (Figure 7).

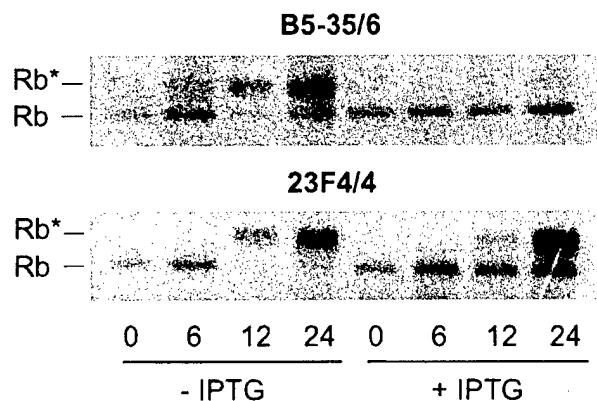
When tested in SETGAP, the full-length Bop1 construct inhibited proliferation of LAP3 cells, indicating that increased levels of Bop1 can induce cell cycle arrest (Figure 7). The effect of Bop1



**Figure 5** Expression of B5-35 or 23F4 inhibits S phase entry in serum-stimulated cells. Clonal lines transfected with either the empty vector (pX11/1), or B5-35 (B5-35/6) or 23F4 (23F4/4 and 23F4/5) were plated in chambers on glass slides and starved in 0.1% serum for 72 h. Cells were re-stimulated with 10% serum in medium containing 10  $\mu$ M BrdU. Cells were either treated (■) or untreated (□) with IPTG (1.5 mM) 6 h prior to serum stimulation, and again at the time of serum addition. At the indicated times following stimulation (12–30 h), cells were fixed and stained with anti-BrdU antibodies. The percentages of BrdU-positive nuclei were determined by observing at least 200 cells at each time point. An independent experiment monitoring [ $^3$ H]thymidine incorporation showed similar results

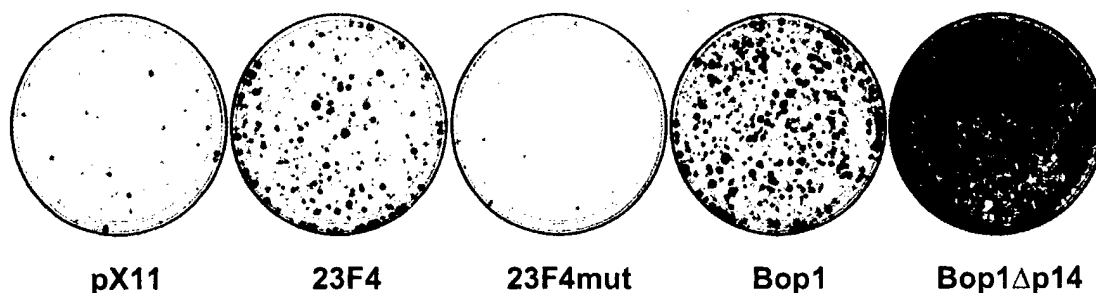
expression, however, was not as striking as that of B5-35. The simplest interpretation of this fact is that the deletion of the amino-terminal part of Bop1 creates a gain-of-function mutant. Another possibility is that the amino-terminal deletion in B5-35 might create a dominant-negative mutant that is more potent in perturbing Bop1 function than overexpression of the full-length protein. Further studies will be needed to determine the physiologic role of Bop1 and to understand how interference with its function results in a strong, albeit reversible, block to cell cycle progression.

Database analyses revealed two sequences that are homologous to Bop1: one in human (KIAA0124), and one in the yeast *S. cerevisiae* (YM9796.02c) (Figure 9). The human KIAA0124 sequence was obtained by sequencing randomly chosen human cDNA clones and contains a partial coding sequence (Nagase *et al.*, 1995). The yeast sequence YM9796.02c was deduced from an open reading frame found in the genome of this organism. All three amino acid sequences are highly homologous except in their amino-termini. The deduced mouse Bop1 shares approximately 45% amino acid sequence identity with the yeast YM9796.02c, and >90% amino acid identity with the human sequence, excluding the amino-terminus (Figure 9). The amino-terminal domains of all three of these proteins are divergent in their primary structure, although they are



**Figure 6** Phosphorylation of Rb is inhibited or delayed in cells expressing B5-35 or 23F4. The cell lines B5-35/6 and 23F4/4 were made quiescent by incubation in 0.1% serum for 40 h. Cells were then stimulated with 10% serum for the indicated times (hours) either in the presence or absence of 1 mM IPTG (added 16 h prior to serum stimulation where indicated). Cell lysate was prepared and Rb was detected by Western blotting (see Materials and methods). Rb\*, hyperphosphorylated form of Rb

very similar in composition. The most prominent feature of these domains is that they are rich in acidic and serine residues and contain PEST sequences, which are often found in short-lived regulatory proteins (Rechsteiner and Rogers, 1996).



**Figure 7** Effects of mutations in UbcM2 and Bop1. Pools of cells stably transfected with either the empty vector (pX11), or expression constructs of UbcM2 (23F4), a cysteine-145 to serine mutation of UbcM2 (23F4mut), the full length *bop1* (Bop1), and 5' deletion of *bop-1* (Bop1 $\Delta$ p14) were analysed in the SETGAP assay. Expression of transfected sequences was induced with IPTG, and cells were subjected to BrdU/light treatment. Growth of surviving cells was restored by IPTG removal and the resulting colonies were stained 9 days thereafter

The amino acid sequence of Bop1 contains several motifs, known as WD40 (or  $\beta$ -transducin) repeats (Figure 8), which are found in a large number of regulatory proteins (Neer *et al.*, 1994; Neer and Smith, 1996) and may be involved in protein-protein interactions (Wall *et al.*, 1995; Lambright *et al.*, 1996; Sondek *et al.*, 1996). The WD40 motif encompasses approximately 40 amino acid residues with several conserved features, including a characteristic Trp-Asp (WD) dipeptide. All WD40 repeat proteins contain from 4 to 10 repeats that display different degrees of divergence from the consensus WD40 motif structure (Neer *et al.*, 1994). Four WD40 repeats were identified in Bop1 by a pattern search against the Blocks database (Petrokovski *et al.*, 1996). Repeats 1 and 4 (Figure 8) are close to the consensus structure, while repeats 2 and 3 are more divergent. Interestingly, repeat 3 contains a Cys residue instead of the canonical Trp (or another aromatic) residue in the marker WD dipeptide, although it gains a high score in a computer search as a true WD40 repeat due to proper positioning of other characteristic amino acid residues.

## Discussion

The SETGAP procedure was developed as a genetic approach to isolate growth-inhibitory sequences based on a functional assay in mammalian cells (Pestov and Lau, 1994). This procedure was first applied in the selection of short genetic suppressor elements targeted at a small group of known growth-related genes. In this report, we describe an adaptation of the SETGAP procedure for the cloning of growth-inhibitory cDNA sequences from a complex library. This adaption takes advantage of the genetic selection conferred by SETGAP to enrich for growth inhibitory sequences, followed by a screening procedure to identify individual clones.

We started by constructing a conditional expression cDNA library using mRNA from mouse fibroblast passaged to crisis; the cDNA library was transfected into LAP3 cells and an estimated  $10^4$  of the transfected clones were first subjected to selection to enrich for growth-inhibitory cDNA sequences. Each of the transfected clones typically contained 10–100 cDNA sequences, as judged by PCR analysis (data not

shown). After two consecutive rounds of SETGAP selection, the PCR pattern of cDNA recovered from cells was simple enough to permit analysis of individual clones.

In this study, we carried out our selection and screening with a relatively small number ( $10^4$ ) of transfected clones. Given that typically 10–100 sequences are incorporated into each clone, the actual number of sequences that have been tested in this screening is much larger than the number of clones screened. However, we speculate that the two growth inhibitory cDNAs we isolated in this screening represents only a small fraction of such sequences in the library for several reasons. First, the transfected sequences must be expressed at an appropriate level under induction to cause transient growth arrest. Second, we encountered a relatively low success rate in recovery of growth-inhibitory cDNA sequences from growth-arrested cells during selection (about 20% in this study). This may be due to a loss of integrity of some cDNA sequences upon chromosomal integration, which could prevent their amplification by PCR. Third, it is possible that in some cells growth arrest is caused by combinatorial or additive action of multiple sequences, and this effect may be lost when the sequences are tested individually. We also noted that some cDNA sequences chosen at random for testing were refractory to amplification by Pfu, although they could be efficiently amplified by other enzymes. To improve the recovery of cDNA sequences from transfected cells, we now use PCR protocols that utilize a combination of polymerases.

Most of the problems that limit the efficacy of the present experimental procedure derive from the nature of transfection in mammalian cells. Perhaps a different method of introduction of cDNA into cells (for instance, using virus-based or episomal vectors) might improve the success rate in the selection step. Some of the aforementioned problems can be addressed by screening a larger number of clones, and in this regard, reduction of the complexity of the starting library should greatly enhance the efficacy of the procedure. Recently, highly efficient methods for normalization of cDNA libraries have been developed (de Fatima Bonaldo *et al.*, 1996). The significantly lower overall complexity of such libraries should make it possible to analyse a large fraction of the cDNAs.

One of our original concerns in application of expression cloning for the isolation of growth-inhibitory cDNA was that this approach might primarily yield cDNA clones that encode proteins that have no clear role in growth control but nevertheless cause growth inhibition when their expression is out of balance relative to other cellular components. In fact, many 'structural' genes (such as actin,  $\beta$ -tubulin, actin-binding protein, nonhistone protein B) were isolated in yeast screens for overexpression-induced growth suppressors (Liu

*et al.*, 1992). However, we have not identified any structural gene sequences in our search for growth inhibitory sequences to date. Screening of small groups of clones (10–100 sequences each) from the original cDNA library, also did not yield such sequences, even though abundant transcripts should be represented among these clones (data not shown). A possible reason for this is that previous screening procedures (such as screens in yeast) identified growth-inhibitory sequences based on induction of a lethal phenotype. In contrast, the SETGAP

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CTCCCGCTGC CGAAGCGGC AGTAGATCGG TAGCCGGCGC GGCGCAGCAT GGCGGGGGCG TGTGGTAAGC CCCACATGTC ACCGGCATCG CTGCCGGGGA 100
      *
      M A G A C G K P H M S P A S L P G
      |
      |  $\alpha$ 8 start
AACGACGTTT GGAACCTGAT CAGGAGCTGC AGATACAAGA GCCTCTCTCT CTCACGATC CTGACTCCAG TCTCTCTGAC AGCGAGGAGA GTGTGTTTTC 200
K R E L E F D Q E L Q I Q E P P L L S D P D S S L S D S E E S V F S
AGGCTCTGAA GATTTGGGCA GTGACTCGAG TGAGGAAGAC ACTGAAGGAG TGGCGGGATC CAGCGGTGAC GAAGACAACC ATAGGGCAGA GGAGACCTCT 300
G L E D L G S D S S E F D T E C V A G S S G D E D M H R A E E T S
GAGGAGCTGG CACAGGCTGC CCCTCTTTCG TCAAGGACAG AGGAAGCAGG TGCTCTGGCC CAAGATGAAT ATGAAGAGGA CAGCTCTGAT GAGGAGGACA 400
E E L A Q A A P L C S R T E E A G A L A Q D E Y E E D S S D E E D
TTCGGAACAC TGTGGGCAAT GTGCCCTTGG CATGGTACGA TGAATTCCTA CATGTGGGTT ATGACCTGGA TGGCAACCGT ATCTACAAGC CCCTGCGGAC 500
I R N T V G N V P L A W Y D E F F H V G Y D L D G K R I Y K P L R T
ACGAGATGAG CTCGACCACT TTCTGGACAA AATGGATGAC CCAGATTCTT GGCGCACTGT GCAAGACAAG ATGACAGGGC GTGATCTGCG GCTAACTGAT 600
R D E L D Q F L D K M D D P D F W R T V Q D K M T G R D L R L T D
GAGCAGGTTG CCCTGGTACA CCGGCTTCAG AGAGGCCAGT TCGGAGATTC AGGCTTCAAT CCCTATGAGC CAGCTGTGGA CTCTCTCAGC GGTGACATCA 700
E Q V A L V H R L Q R G Q F G D S G F N P Y E P A V D F F S G D I
      |
      | B5-35 start
TGATCCACCC TGTGACCAAC CGCCCACTGC ACAAGCGTAG TTTCATCCCA TCCCTAGTTG AAAAGGAGAA GGTGTCTCGA ATGGTACATG CCATCAAGAT 800
M I E P V T N R P A D K R S F I P S L V E K E K V S R M V H A I K M
GGGTGGGATC AAGCTCTGAC GGCCCCACGA CCCACCCCTC AGCTTCTATG ACCTGTGGGC CCAGGAGGAT CCGAATGCTG TGTGGGGAGC CCACAGATG 900
G W I K P R R P H D P T P S F Y D L W A Q E D P N A V L G R H K M
CATGTGCTGC CCCCAAGCT GGCCCTTCCG GGCCATGCGC AGTCTTACAA CCCACTCCC GAGTACCTGC CCACTGAGGA GGAGCGCTGC GCTGGGATGC 1000
H V P A P K L A L P G H A E S Y N P P P E Y L P T E E E R S A W M
AGCAGGAGCC TGTGGAGAGG AAGCTTAACT TCTTGCCACA GAAATTCCTC AGCTTGAGGA CAGTGCCTGC TTACAGCCGC TTCATCCAGG AGCGTTTCGA 1100
Q Q E P V E R K L N F L P Q K F P S L R T V P A Y S R F I Q E R F E
GCGCTGCCCT GATTGTATC TGTGCCACG GCAACGCAAG ATGAGGGTGA ATGTGGACCC CGAAGACCTC ATCCCCAAC TCCCTCGGCC AAGAGACCTT 1200
R C L D L Y L C P R Q R K M R V N V D P E D L I P K L P R P R D L
CAGCCTTTC CTGTGTGCCA GGCCCTCTGC TACAGGGGCG ACAGCGACCT TGCTCGTTCG CTCAGTGTCT CGCCAGGGGG CCAGTGGCTA GCTTCAGGTT 1300
Q P F P V C Q A L V Y R G H S D L V R C L S V S P G G Q W L A S E G
CAGCAGTATG CACGTGGAAG CTCTGGGAGG TGGCCACTGC CCGCTGTATG AAGACTGTAC ATGTGGGAGG TGTGGTGGCG AGCATCGCCT GGAACCCCAA 1400
S D D G T D K L W E V A T A R C M K T V H V G G V V R S I A W N P N
TCCTACCATA TGCTGGTAG CTGCTGCCAT GGATGATGCC GTGTGTCTGC TGAACCCAGC CCGGGGGGAC GCGGTCTGCG TGGGCAGCAC AGACCAGCTG 1500
P T I C L V A A A M D D A V L L L N P A L G D R L L V G S T D Q L
CTGGAAGCCT TCACTCCACC TGAGGAGCCA GCTCTGACG CTGCCCGGTG GCTAGAGGTC TCGGAGGAAG AGCACCAGAG GGGTCTGCGC CTACGCATCT 1600
L E A F T P P E E P A L Q F A R W L E V S E E E H Q R G L R L R I
GCCACAGCAA ACCAGTGACA CAGGTGACCT GGCATGGGCG AGGGGACTAT TTGGCGGTGG TGCTGTCTAG TCAAGAGCAC ACGCAAGTGC TGCTTACCA 1700
C H S A P V T Q V T W H G R G D Y L A V V L S S Q E H T Q V L H Q
GGTGAGCAGG AGGCGCAGCC AGAGCCCGGT CCGCCGCGAG CACGGGCGAG TGCAGTGTGT GGCCTTCCAT CCCTCCCGGC CCTTCTGCT CGTGGCCTCC 1800
V S R R R S Q S P F R R S H G Q V Q C V A F H P S R P F L L V A S
CAGCGCAGCA TCCGCATTTA CCACCTGCTG CGCCAGGAGC TAACCAAGAA GCTGATGCCC AACTGCAAGT GGGTGTCCAG CATGGCTGTA CATCCAGCAG 1900
Q R S I R I Y H L L R Q E L T K K L M P N C K W V S S M A V E P A
GTGACAACAT CATCTGTGCG AGCTATGACA GCAACTGGT GTGGTTTGAC CTGGATCTTT CCACCAAGCC ATACAAAGTG CTGAGGCACC ACAAGAAGGC 2000
G D N I I C G S Y D S K L V W F D L D L S T K P Y K V L R H H K K A
CTTGGGGCT GTGGCTTTC ACCCCGATA CCCACTCTT GCATCGGGCT CAGACGAGCG CAGTGTATC GTGTGCCATG GCATGGTGTG CAATGACCTT 2100
L R A V A G F E A S G S A D G S V I V C H G M V Y N D L
CTGCAGAACCAT CATGTGGTGT GCCTGTCAAG GTGCTGAAG GACACACCT GACCCGAGAT CTGGGTGTTT TGGATGTGGC CTTCACCCCC ACACAGCCGT 2200
L Q N P L L V P V K V L K S H T L T R D L G V L D V A F H P T Q P
GGGTCTTCTC CTCCGGGGCA GATGGCAACA TTCGACTCTT CAGCTAAGCC AACACACTAC TCTGGGTGGC CTGGAAGAGG GTGGGTGTGC CTCCACACAG 2300
W V F S S G A D G T I R L F S *
AATCTCAAT AGAATCCCAT TCTCTCTGTC TTCCTGATTC AGCCTCCAGC AACTGCCAGC CCTTGACCTT ACACAGCACC CAGAACCTGG CTTAAGCCAG 2400
AAGGGAAGGT GGTATTGATA CAATGTCAAC CACTGAGGTG TAATGGGCTT TATTGGGCAA TAAATTGGGG CACCACAARA AAAAAAAAAA AAAAA
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Figure 8 Nucleotide and deduced amino acid-sequences of the *bop1* cDNA. The full-length *bop1* cDNA encodes a protein of 732 amino acids containing four WD40 repeats (underlined). The PEST sequence in the amino-terminus is underscored by a dash line. The starting positions of clones B5-35 and  $\alpha$ 8 are indicated by arrows. The two ATG codons at the start of the B5-35 open reading frame are at nt 781 and 799. (T) denotes a single-base substitution found in B5-35 when compared to the sequence of clone  $\alpha$ 8 isolated from the cDNA library. Asterisks indicate in-frame stop codons

method makes use of the formation of colonies by surviving cells, which requires two events: (i) stringent blockade of DNA replication upon induction of transfected sequences, lasting for the duration of BrdU treatment (typically 48 h), and (ii) reversal of the growth arrest when expression of transfected sequences is downregulated. Hence, factors that can slow down metabolism but incapable of causing a prolonged cell cycle block or sequences that are toxic for a cell would not be identified in SETGAP. We anticipate that this feature of the SETGAP method should facilitate the identification of regulatory genes. In support of this idea, tests with several known genes capable of arresting the cell cycle in a reversible manner showed that they can be readily identified by the

SETGAP procedure (for example, see results with p27Kip1, Figure 2b).

The two growth-inhibitory cDNAs isolated in this study were 1.2 and 1.75 kb in length. Since the majority of the mRNAs in the cell are between 1–2 kb, we expect that many growth-inhibitory cDNA sequences can be successfully recovered using the present experimental protocol. One of the isolated clones, 23F4, encoded a full coding sequence of UbcM2, while clone B5-35 contained a partial deletion of Bop1 cDNA, which fortuitously created a very strong growth-inhibitory sequence. The isolation of partial cDNA fragments is not surprising, as many clones in any cDNA library are products of incomplete cDNA synthesis. Although this may initially appear a disadvantage, the isolation of

Bop1	: MAGACGPHMSPASLPCHLELPDTELQIEPPLSDPDSSSLDSEESVFSHEELGSSSEELTEGVASSGDFDNHRA	: 80
KIAA0124	: -----SGSSSEEDDGGEGEDADDDHGGGI	: 30
YM9796.02c	: ---MMAENNKTEAKMSRAASESDVEDEDKILS-----VGGIIAEASEEDDEDEYESAEEKESSD	: 65
Bop1	: EETSEFLAAPLSTHEAGLAQD-----E	: 109
KIAA0124	: KTTETQVASTPPTTEHSSARIGD-----A	: 59
YM9796.02c	: EAQDDSDDDDAELNKLLEEGDGEDYDSSEFSDDTSLTDLRSGVKLQTIQVDPNIYSKYADGSDRIIKPEINPVYD	: 145
Bop1	: EDSSDEEIRITVGVVPLATYDEFFHVGYDILGFFIYKPLTRDELEELFLDEHDDPDEFWTVQDEHTIGEBLRLTDEQVAL	: 189
KIAA0124	: EDSSDEEIRITVGVVPLATYDEFFHVGYDILGFFIYKPLTRDELEELFLDEHDDPDEFWTVQDEHTIGEBLRLTDEQVAL	: 139
YM9796.02c	: SLDSEIATQITIGMTESATIDMMHTGYDINGFFIMPAAG-SALDGLSHELEEGATGLDINSASSLNLRKELEI	: 223
Bop1	: NRRLEGGQFGSSGFHPYEPADDFSGT---IMIEVTHRPADKRSFIPSLVPRERKVSPPVHALENGHKK-----RKTH	: 260
KIAA0124	: NRRLEGGQFGHVGHPYEPADDFSGT---IMIEVTHRPADKRSFIPSLVPRERKVSPPVHALENGHKK-----RRMR	: 210
YM9796.02c	: LSKIQFNEQTDSIHPYELLIDWTRHEEVI---PLTAVTEPEERFVPSKNLAKRYMTIVRAIPEERTIEPKKLKEMEKE	: 301
Bop1	: DPTSEYDLAAQEGPHAVLGRHFNHVPAPKIALPGLHAESTYPPPEYIETEESSARMQOEFVRRRTNPLPQTFPSLPTVF	: 340
KIAA0124	: DPTSEYDLAAQEGPHAVLGRHFNHVPAPKIALPGLHAESTYPPPEYIETEESSARMQOEFVRRRTNPLPQTFPSLPTVF	: 290
YM9796.02c	: KIENYQIDLG---DSTETNDVHLRAPHPPPTNEESYHDPYELLSPEEHEAKENTYSEPERFIPOKYSALRYKF	: 378
Bop1	: NTSRAIQERIEKLDLITCFRQFTHRVVDFEDLITPLPFRDIDQITVQALVYFGHSDLVRCISVSIQCCQWLASGSD	: 420
KIAA0124	: NTSRAIQERIEKLDLITCFRQFTHRVVDFEDLITPLPFRDIDQITVQALVYFGHSDLVRCISVSIQCCQWLASGSD	: 370
YM9796.02c	: GCGESIRREFERSLGLYIADVPINPLHIDNSLITELTSEPTIRPFPFRCSTIATKGRMPTTSDNSLWLTATGSD	: 458
Bop1	: SFLRLWEVATARCMTYHVG-----VYRSTANHHETITCLVAAADAPALLH-----ALGDRI	: 476
KIAA0124	: SFLRLWEVATARCMTYHVG-----VYRSTANHHETITCLVAAADAPALLH-----ALGDRI	: 426
YM9796.02c	: STVRVREITIGELYRRTLIDDEENPDYHECTEHNPIANNGLIAYVNGENITLIVPIFGYDIENNGTKIEDGFFYDT	: 538
Bop1	: LVGSTDQLLEAHTPE---EEPA-----LQPARWLEVEEHHDPGLRLRICHSPVTVQVTHGRGDYLAVALSSCEH	: 544
KIAA0124	: LVGSTDQLLEAHTPE---EEPA-----LQPARWLEVEEHHDPGLRLRICHSPVTVQVTHGRGDYLAVALSSCEH	: 494
YM9796.02c	: FGTVKKSNLAVNENGDDGDEGENESAKNAVKKVFQCNKPKKLLDLCITISCKETKKLSWHREGDYFTVQPDSEN	: 618
Bop1	: FQVLLHQVSKRSDSPFEDHGVCVAFHISPPFLLVASQSPFIRIHLRQELTFFELHPCFVSSSAVHPAGDNIIICG	: 624
KIAA0124	: FQVLLHQVSKRSDSPFEDHGVCVAFHISPPFLLVASQSPFIRIHLRQELTFFELHPCFVSSSAVHPAGDNIIICG	: 574
YM9796.02c	: ISVLLHQVSPHITQSPFFSKITIMDARFHEFFHQVYCBQFYRINDSQITVIFLLGAWLKKIDHERGDHLLAS	: 698
Bop1	: SYDSRLVWFDDLLSTFPTVHLNHHKFAIYAVAFHPTPLFASGSDGSSVIVCHGMVYNDLLQNPPLVPPVFLVLEGGHTIRE	: 704
KIAA0124	: SYDSRLVWFDDLLSTFPTVHLNHHKFAIYAVAFHPTPLFASGSDGSSVIVCHGMVYNDLLQNPPLVPPVFLVLEGGHTIRE	: 654
YM9796.02c	: STIKRVIMHDLDAETTYTLYNDEAVFSNFKKIPLESAAADDGTHFATATYDNNKIPNIVPLIKITGKRVINS	: 778
Bop1	: LGVLDVAFHPTOPWVFSSGADGTILFE-----	: 732
KIAA0124	: LGVLDVAFHPTOPWVFSSGADGTILFE-----	: 682
YM9796.02c	: LGVLDVAFHPTOPWVFSSGADGTILFE-----	: 807

**Figure 9** Comparison of amino acid sequences of the murine Bop1 and its human (KIAA0124) and yeast (YM9796.02c) homologs. Alignment was compiled using the Clustal W program (Thompson *et al.*, 1994) and modified by using GeneDoc (Nicholas *et al.*, 1997). Shading indicates identical positions and conservative substitutions

truncated cDNA sequences in a selection for growth inhibition can also be informative. For instance, identification of dominant negative variants of regulatory genes essential for cell replication could reveal practical ways to inactivate such genes. It is conceivable that some partial cDNAs isolated by this method could encode activated forms of growth-inhibitory proteins. In this regard, we note that many of the genes identified by their ability to induce cell transformation in traditional gene transfer experiments represent activated forms of their normal cellular variants.

The specific cellular processes affected by overexpression of the ubiquitin-conjugating enzyme UbcM2 (clone 23F4) that can lead to inhibition of cell cycle progression are currently unknown. The ubiquitin degradation system is known to regulate a variety of cellular processes (Liu *et al.*, 1992; Hochstrasser, 1995), including the turnover of many proteins that control cell growth, including cyclins (Seufert *et al.*, 1995), c-Myc (Ciechanover *et al.*, 1991), p53 (Scheffner *et al.*, 1993; Maki *et al.*, 1996) and others (Bai *et al.*, 1996; Schneider *et al.*, 1996). UbcM2 belongs to a group of evolutionarily conserved ubiquitin-conjugating enzymes of the E2 class with different amino-terminal extensions (Matuschewski *et al.*, 1996). Overexpression of UbcM2 in yeast can partially suppress deficiency of UBC4 and UBC5 (Hochstrasser, 1995), which are involved in stress response and degradation of regulatory and abnormal proteins (Seufert and Jentsch, 1990; Chen *et al.*, 1993). These data suggest that enzymatic activity of UbcM2 could affect degradation of a regulatory protein(s) whose activity is critical for cell cycle progression.

The B5-35 sequence encodes a fragment of a novel WD40-containing protein, Bop1, truncated from the amino-terminus. Interestingly, expression of this truncated form caused a stronger cell cycle arrest than overexpression of the full-length cDNA. Additional studies will be needed to determine the mechanism of the growth-inhibitory activity associated with expression of Bop1 and whether the B5-35 clone acts by a dominant-positive or negative mechanism with regard to the full-length clone. Several lines of observation suggest that Bop1 may function as a regulatory protein. First, all other WD40 proteins studied to date have been implicated in regulatory activities (Neer *et al.*, 1994; Neer and Smith, 1996). Second, the presence of PEST sequences suggests that Bop1 may be short-lived, also pointing to possible regulatory functions (Rechsteiner and Rogers, 1996). Finally, the structural conservation of Bop1 from yeast to human suggests that function of this protein may also be conserved in diverse eukaryotic cells.

In summary, we have developed a method for the isolation of growth-inhibitory cDNAs from cDNA libraries. This experimental procedure could find application in identification of new cell cycle inhibitors and discovery of antiproliferative genes that may regulate the resting state in quiescent and senescent cells. This genetic strategy could potentially yield a class of growth-inhibitory molecules that would be difficult or impossible to isolate based on biochemical studies of known regulatory pathways.

## Materials and methods

### Cell culture

Cell culture. LAP3 cells (Pestov and Lau, 1994), an NIH3T3 cells-derived cell line which constitutively expresses the IPTG-regulated transactivator protein LAP267 (Baim *et al.*, 1991), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone) and penicillin-streptomycin (GIBCO-BRL). Cells were transfected by the calcium phosphate coprecipitation method (Chen and Okayama, 1988) using 2.5 µg of library DNA or 0.5 µg of a specific construct, 0.5 µg of pHyg (Sugden *et al.*, 1985) and 2.5–4.5 µg of carrier NIH3T3 DNA per 60-mm dish. Stable clones were selected in 130–150 µg/ml<sup>-1</sup> hygromycin (Boehringer Mannheim). Expression of transfected constructs was induced by the addition of 1–1.5 mM IPTG (dioxane-free, Sigma) to culture medium.

### Plasmids

The inducible vector pX11 designed for low background expression, the inducible luciferase reporter pX8B6-luc and growth-inhibitory clone 11-11 (a fragment of *junB*) have been described previously (Pestov and Lau, 1994). The murine cyclin/cdk inhibitor p27(Kip1) was cloned into pX11 to generate pX11-p27; induction of this construct with IPTG inhibited growth of LAP3 cells (Shiyanov *et al.*, 1997).

### Construction of the inducible cDNA library

Mouse embryo fibroblasts were derived from gestational day 17 embryos of Swiss mice by standard techniques (Loo *et al.*, 1989). The cells were passaged according to the 3T3 regimen (Todaro and Green, 1963) in DMEM containing 10% calf serum and penicillin-streptomycin. Cells were counted at each passage to monitor the decline in replicative potential. At the beginning of the crisis stage, their RNA was isolated by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Poly(A) RNA was purified using PolyATtract System (Promega).

cDNA synthesis was carried out using reagents from the SuperScript system (GIBCO-BRL) with several modifications to the manufacturer's protocol. Prior to cDNA synthesis, two oligonucleotides, N3 (CTAGCTGCGGACGAACATTACA) and BR3 (CCGGAATTCGCATGAGATATA), were designed to incorporate partial *NheI* and *BspEI* sites (underlined) into the 5' and 3' ends of the cDNA, respectively. These primers were later used in PCR rescue of cDNA sequences from transfected cells. The first cDNA strand was synthesized from 5 µg of poly(A) RNA using 200 pmol of GA-BR3-T16 primer ((GA)<sub>12</sub>TCCGGAATTCGCATGAGATATA(T)<sub>16</sub>). A nucleotide mixture containing 10 mM each of dATP, dGTP and dTTP (Pharmacia) and 6 mM of 5-methyl-dCTP (Boehringer Mannheim) was used instead of the 10 mM dNTP mixture supplied with the SuperScript kit. After the first strand synthesis, dCTP was added to 400 µM for the second strand synthesis. Ligation of double-stranded cDNA with N3 adapters and phosphorylation were performed according to the SuperScript system protocol. The cDNA was then digested with *KpnI*21 (an isoschizomer of *BspEI* that does not cut methylated DNA), extracted with phenol-chloroform and size fractionated on a Sephacryl S-500 column supplied with the kit. The resulting cDNA was ligated into pX11 digested with *NheI* and *BspEI*. Approximately 8 × 10<sup>5</sup> bacterial colonies were obtained by electroporation of DH10B cells (GIBCO-BRL) with the ligated cDNA. The colonies from 35 plates were scraped into 100 ml of SOB medium and incubated with shaking at 30°C for 1 h. The plasmid DNA was isolated using standard techniques.



The library was estimated to contain  $>5 \times 10^5$  clones with an average insert size of 1.2–1.5 kb.

#### *BrdU/light selection of growth-arrested cells*

$10^5$  stably transfected LAP3 cells were plated into a 10-cm dish in medium containing 1 mM IPTG to induce expression of sequences cloned into pX11. After 24 h, BrdU (Sigma) was added to a final concentration of 10  $\mu$ M and cells were cultured for 2 days. Hoechst 33258 (Sigma) was then added to 10  $\mu$ g/ml<sup>-1</sup> for 1 h and cells were irradiated with visible light for 10 min as described previously (Pestov and Lau, 1994). Alternatively, a 10 min incubation with 1  $\mu$ g/ml<sup>-1</sup> Hoechst 33342 (Calbiochem), which was as effective as the longer treatment with Hoechst 33258, was used. The medium was removed after irradiation and cells were rinsed and refed with fresh medium. Medium was changed on days 2 and 4 after irradiation to remove dead cells.

#### *Rescue of transfected DNA from cells after selection*

Cells that survived BrdU/light selection were grown for 8–10 days until they formed small colonies. Genomic DNA was isolated using DNAzol reagent (Molecular Research Center, Inc.) followed by additional treatment with RNase A prior to PCR amplification with Pfu (Stratagene), a high fidelity polymerase (Lundberg *et al.*, 1991; Bej and Mahbubani, 1994). DNA was first amplified using M5 and P3 primers, which flank the cloning site in the pX11 vector (Pestov and Lau, 1994), and then reamplified with N3 and BR3 primers. PCR was carried out in a Robocycler (Stratagene) using the following program: initial denaturation at 99°C for 10 s; cycling at 99°C for 20 s, 50°C (M5 and P3 primers) or 55°C (N3 and BR3 primers) for 40 s, 68°C for 6 min. These parameters allowed efficient amplification of sequences of up to 4 kb. After 16–18 cycles of PCR with M5 and P3 primers, 1–2  $\mu$ l from this reaction was directly used in a second-step PCR with N3 and BR3. The number of cycles in this PCR was determined empirically for each template, since it dramatically affected the outcome of the reaction. Increasing the number of cycles often shifted the bias of amplification toward smaller fragments. Although supplying additional Pfu polymerase (1.25 U per 25  $\mu$ l reaction) helped alleviate this problem, it was important to keep the number of cycles to a minimum, enough to only visualize DNA after electrophoresis of a 5  $\mu$ l aliquot (1/5–1/10 of the total reaction volume) on an agarose gel.

PCR products were extracted with phenol-chloroform and purified using Microcon-100 concentrators (Amicon), followed by treatment with T4 polynucleotide kinase and then with *exoIII* (Kaluz *et al.*, 1992) to expose *NheI* and *BspEI* ends. This DNA was separated on a low melting point agarose gel; fragments larger than 700–800 bp were purified from the agarose using GELase (Epicentre) and ligated into pX11 DNA that had been digested with *NheI* and *BspEI* and dephosphorylated. For isolation of secondary libraries,  $>10^4$  bacterial colonies obtained by electroporation of DH10B cells were scraped from plates, and processed as described above for the primary library. Individual clones were isolated using Qiawell-8 system (Qiagen).

#### *Screening for growth-inhibitory clones ('SETGAP-2')*

LAP3 cells grown in a 6-well cluster were cotransfected with a cDNA clone to be tested and pGK- $\beta$ -gal. After transfection, cells were kept in the same well. After 5–6 days, cells were trypsinized and split into two dishes (1/5 of cells from one well to one 100 mm dish). The waiting period before starting the assay was necessary to relieve non-specific growth inhibition immediately after

transfection. One dish was kept without further treatment and used to control the transfection efficiency by staining the cells with X-gal. To the second dish, IPTG was added and BrdU/light treatment was performed as described above. Five or 6 days after this treatment, cells were stained with X-gal. Cells were washed once with PBS, fixed with cold 0.5% glutaraldehyde in PBS, rinsed twice with PBS and incubated for 2–3 h at 37°C in staining solution (1 mM MgCl<sub>2</sub>, 3.3 mM of each K<sub>4</sub>Fe(CN)<sub>6</sub> and K<sub>3</sub>Fe(CN)<sub>6</sub>, 0.1% X-gal and 0.02% NP-40 in PBS).

#### *Other analytical methods*

To analyse Rb phosphorylation, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8), 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma) for 30 min at 4°C followed by centrifugation for 10 min at 10 000 g. Lysates (~50  $\mu$ g of total protein) were first concentrated by immunoprecipitation and then analysed by immunoblotting using the G3-245 antibody (Pharmingen). Detection of secondary horseradish peroxidase-coupled antibodies (Amersham) was performed with the LumiGLO reagents (KPL).

For immunohistochemical determination of BrdU incorporation, cells cultured in Falcon Culture Slides were fixed with cold 70% ethanol and allowed to air-dry. Slides were rehydrated in water for 30 min, treated with 2N HCl for 30 min, rinsed with 0.2 M Tris-HCl (pH 8), PBST (0.2% Tween-20 in PBS), blocked with 10% fetal calf serum in PBST for 10 min, washed twice with PBST, incubated for 1 h with peroxidase-conjugated anti-BrdU antibodies (Boehringer Mannheim), washed three times with PBST, stained using DAB-Plus kit (Zymed), counterstained with hematoxylin and covered with 70% glycerol. The percentage of BrdU-labeled cells was determined using a light microscope.

#### *Cloning and sequence analysis*

Nucleotide sequences were determined using Sequenase (USB) and EXCEL (Epicentre) reagent kits and protocols. To clone the full-length Bop1 cDNA, the cDNA library described above was screened with a B5-35 probe, and the longest isolated clone of 2345 bp (designated  $\alpha$ 8) was sequenced. Both B5-35 and  $\alpha$ 8 clones contained poly(A) tails at their 3' ends at almost identical positions (six extra nucleotides were present in B5-35). The sequence of B5-35 differed from the corresponding fragment of the library-derived clone  $\alpha$ 8 by a single base substitution (C→T at nt 791 resulting in a Ala→Val change; Figure 8). The 5' end of the cDNA was cloned using the 5' Rapid Amplification of cDNA ends (RACE) System (GIBCO BRL). The sequence of  $\alpha$ 8 was combined with 131 bases derived by sequencing several independent 5' RACE clones into a sequence of 2476 bp. This sequence is likely to contain the full coding sequence since it corresponds in size to the Bop1 transcript, contains an in-frame initiating ATG and in-frame termination codon upstream. The sequences of clone 23F4 and full-length Bop1 have been deposited into the Genebank (accession numbers AF003346 and U77415, respectively). An expression construct for Bop1 $\Delta$  was created by using PCR primers to synthesize the *bop1* cDNA that initiated with nt 799 of the *bop1* sequence (Figure 8), resulting in a 5' truncation. The sequence of the truncated *bop1* was confirmed by sequence determination, and cloned in pX11. A cysteine-145→serine mutation (23F4Mut) in UbcM2 was created by a T→A transversion at nt 436 of the 23F4 sequence.



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# IPTG-Inducible Episomal Expression System for Exogenous Genes in Primate Cells

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## ABSTRACT

An isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG)-inducible episomal expression system has been established for the human breast carcinoma cell line MCF7. This two-component system includes: (i) a primate cell-specific episomal vector, pEpiLac, that contains an IPTG-inducible promoter and (ii) a cell line derived from MCF7, MCF7/LAP5, which expresses the IPTG-dependent transactivator LAP267. Treatment of MCF7/LAP5 cells with IPTG results in efficient inducible expression of exogenous genes from the inducible promoter in pEpiLac. Up to 300-fold induction can be observed when luciferase is used as a reporter. Inducible expression of the p27<sup>KIP1</sup> cyclin-dependent kinase (CDK) inhibitor, the orphan nuclear receptor Nur77 and the angiogenic inducer Cyr61 has also been demonstrated. Expression of the exogenous gene is promptly halted on removal of IPTG. Moreover, the episomal vector can be stably maintained in and easily recovered from MCF7/LAP5 cells. Taken together, this inducible expression system should be applicable for the regulated expression of exogenous genes, especially growth inhibitory or cytotoxic genes, in cells of primate origin.

## INTRODUCTION

The ability to express an exogenous gene in an inducible manner in mammalian cells is often desirable, especially when the gene product is potentially cytotoxic or growth-inhibitory. To this end, several inducible expression systems have been established. Among them is the isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG)-inducible expression system, which consists of an expression vector with a basal promoter linked to the *Escherichia coli* lac operator sequences (3). Genes cloned under the control of this inducible promoter can be specifically activated by the IPTG-dependent transactivator LAP267, a chimeric protein containing the herpes simplex virus virion protein 16 (VP16) transactivation domain linked to the *E. coli* lac repressor (3). LAP267 functions as a temperature-sensitive transactivator, being active at 32°C but inactive at the normal cell growth temperature of 37°C (3). However, its activity is restored by the presence of IPTG at the nonpermissive temperature (3).

Recently, this IPTG-inducible expression system has been modified, and its background level of expression was reduced (17). A modified mouse mammary tumor virus (MMTV) basal promoter was put in place of the simian virus 40 (SV40) early promoter used in the original vector. Two Oct-1 binding sites within the basal MMTV promoter were destroyed by mutation, resulting in very low basal or leaky expression from the inducible promoter (15). This vector system is efficacious for inducible gene expression in transfected

mammalian cells (16).

We have sought to improve this inducible vector system further by incorporating the advantages of an episomal vector. The presence of the Epstein-Barr virus (EBV) origin for plasmid replication (*oriP*) and the EBV nuclear antigen 1 (EBNA 1) gene on the same DNA molecule allows for extra-chromosomal replication in a variety of established primate cells, but not in rodent cells (20). Such EBV-derived plasmids replicate only once per cell cycle and are not amplified on entry into cells (19); they are maintained at anywhere from a few copies to about one hundred copies per cell under selection in different cell lines, and are lost from the cell population at rates of about 5% per generation in the absence of selection (19,20).

Episomal vectors such as those derived from the EBV replicon have several advantages (8,11,12,14). First, the absence of integration into host-cell chromosomes obviates potential problems of integration position effects on gene expression and reduces the likelihood of any rearrangement of the transfected DNA (8,11,12,14). Second, the expression levels of transfected genes are consistently higher than expression from non-episomal vectors (11). Third, recovery of the transfected episomal plasmids is simple and efficient using the Hirt's extraction procedure (4,9).

Here, we report the construction of an IPTG-inducible episomal expression vector, pEpiLac, which combines the advantages of both the IPTG-inducible expression system and the EBV-based, extra-chromosomal replication system.

We also introduced the LAP267 transactivator into the human breast carcinoma cell line MCF7, thus establishing a MCF7-derived cell line in which the pEpiLac vector can efficiently express exogenous genes in an inducible manner.

## MATERIALS AND METHODS

### Cell Culture

MCF7 cells and MCF7/LAP5 cells were maintained at 37°C and 5% CO<sub>2</sub> in MEM containing nonessential amino acids, plus 1 mM sodium pyruvate, 1 mM glutamine (all from Life Technologies, Gaithersburg, MD, USA), 10% fetal bovine serum (Intergen, Purchase, NY, USA) and 10

µg/mL bovine insulin (Life Technologies). Cells were refed every 2–3 days. Where indicated, hygromycin B (Roche Molecular Biochemicals, Indianapolis, IN, USA) or G418 (Life Technologies) was added into the medium to the final concentrations of 75 µg/mL or 600 µg/mL, respectively. No G418 selection pressure is required to maintain MCF7/LAP5 cells.

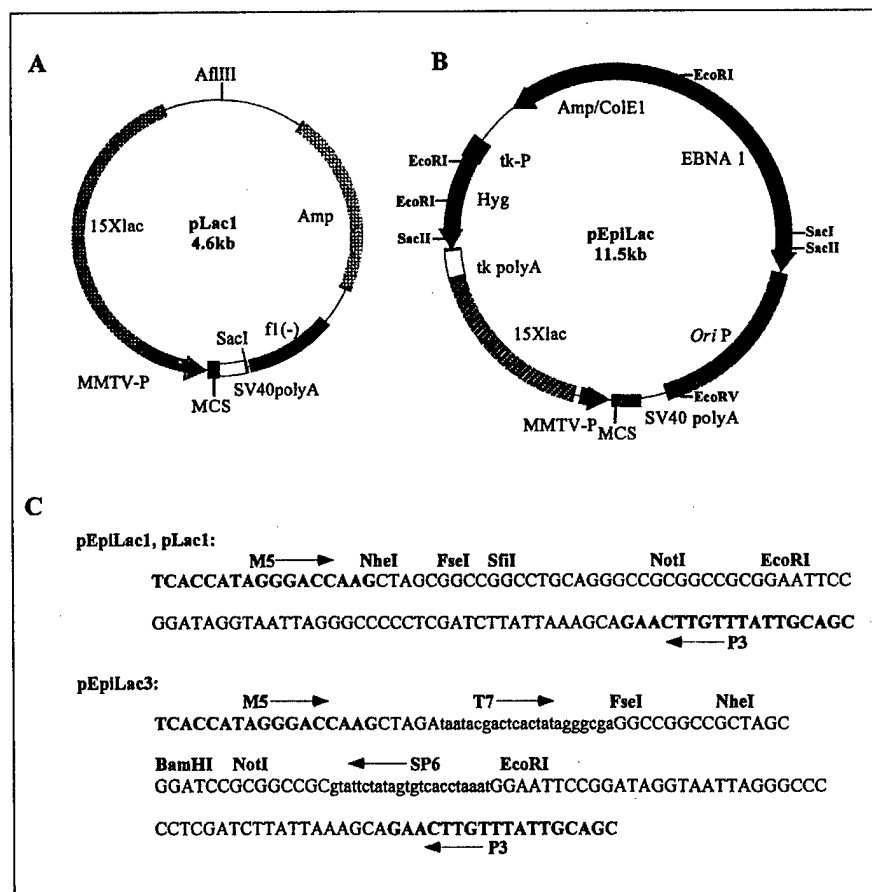
### Transfections

For electroporation,  $2 \times 10^7$  cells in 0.8 mL PBS, pH 7.35, and 20 µg DNA were incubated at room temperature for 10 min, electrically pulsed in a 0.4 cm cuvette (Bio-Rad Laboratories, Hercules, CA, USA) at 960 µFD and 330 V. After 5 min incubation at room tem-

perature, cells were cultured in fresh medium. For calcium phosphate precipitation, 7.5 µg total DNA and  $5 \times 10^5$  cells were used for each 60 mm plate. Cells were exposed to DNA-calcium phosphate precipitates for 8 h and glycerol-shocked (15% glycerol in 20 mM HEPES-K<sup>+</sup>, pH 7.2) at room temperature for 1 min. Glycerol was removed by washing cells twice with PBS. Thereafter, cells were cultured in fresh medium, and IPTG was added where indicated. In all transfections, pSG5 (Stratagene, La Jolla, CA, USA) DNA was used as carrier to bring up the total DNA amount. For stable transfections, cells were split [1 to 5] into hygromycin B or G418-containing mediums two days after transfection and further selected for 3 weeks.

### Construction of pEpiLac

An IPTG-inducible episomal expression vector, pEpiLac1 (Figure 1B), was constructed from the episomal vector pREP4 (Invitrogen, Carlsbad, CA, USA) and the pLac1 vector (Figure 1A). pLac1 was derived from pX12 (15), with a multiple cloning site (MCS) that was modified by the insertion of a pair of oligonucleotides containing three rare-cutting restriction enzyme recognition sites: *FseI*, *SfiI* and *NotI* (Figure 1C). pLac1 was double-digested with *AflIII* and *SacI* and the cohesive ends were filled-in using Klenow DNA polymerase (Promega, Madison, WI, USA). pREP4 was digested with *SaII* followed by Klenow DNA polymerase treatment. The large fragment from pREP4 contains the machinery for DNA replication as well as EBNA 1 and EBV *oriP*. The small fragment from pLac1 contains the IPTG-inducible MMTV basal promoter, MCS and the SV40 polyadenylation signal. pEpiLac1 resulted from the ligation of these two fragments (Figure 1B). pEpiLac3 was derived from pEpiLac1 by modifying the MCS (Figure 1C). Hygromycin B is required for long-term maintenance of cells that are transfected with this episomal vector because episomal vectors do not integrate into host cell chromosomes; in the absence of selection, episomal vectors are lost from the cell populations at rates of close to 5% per generation (19,20).



**Figure 1. Schematic representation of IPTG-inducible expression vectors.** The physical maps of the pLac1 non-episomal vector (A) and the pEpiLac episomal vector (B) are shown. Sequence of their MCS are shown in panel C. Abbreviations are: *OriP*, origin for plasmid replication of EB virus; EBNA1, EB virus nuclear antigen 1; Hyg, hygromycin B resistant gene; lac, lactose operator sequences; MMTV-P, mouse mammary tumor virus basal promoter; SV40 polyA, SV40 virus polyadenylation signal; M5 and P3, PCR primers used to amplify inserts in the MCS; T7 and SP6, promoters that transcribe DNA sequences in sense orientation and antisense orientation, respectively. *FseI*, *SfiI* and *NotI* are rare-cutting restriction enzyme sites.

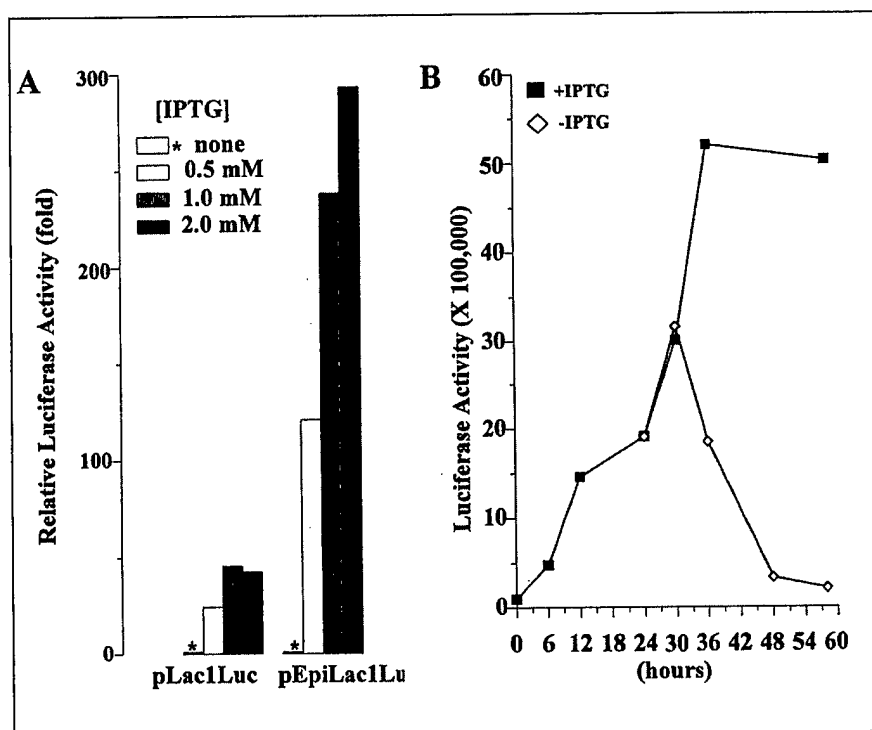
## Extraction of Episomal DNAs

Episomal DNAs were isolated from MCF7/LAP5 cells using Hirt's extraction method (4,5) with some modifications. Briefly,  $1-2 \times 10^6$  cells were harvested in TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl.) washed once with cold PBS and then resuspended in 0.36 mL ice-cold TE buffer (10 mM Tris-HCl, pH 7.2, and 10 mM EDTA). SDS was added to the cell suspension for a final concentration of 1% and incubated at room temperature for 10–20 min after gentle mixing. One-fifth volume of 5 M NaCl was then added, gently mixed and incubated at 4°C overnight. Samples were centrifuged at  $15\,000\times g$  for 30 min at 4°C. The supernatant was collected and extracted once each with phenol, phenol:chloroform (1:1), and chloroform:iso-

amyl alcohol (24:1). Episomal DNA was precipitated with 0.3 M sodium acetate and 2–3 volume of absolute alcohol and dissolved in distilled water.

## Protein Assays

For Western blot analysis, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet<sup>TM</sup> P-40 (Sigma, St Louis, MO, USA), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1% aprotinin and 10 µg/mL leupeptin) on ice for 10 min, centrifuged at  $15\,000\times g$  for 15 min at 4°C. Protein were quantified using Dc protein assay reagents (Bio-Rad Laboratories), separated in 10% SDS-PAGE and electroblotted. Proteins were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using



**Figure 2. IPTG-induced expression of the luciferase reporter gene in MCF7/LAP5 cells.** (A) MCF7/LAP5 cells were transiently cotransfected with pPGKβGal (1 µg) together with either pLac1Luc (2 µg) or pEpiLac1Luc (3 µg, about 25% less in molar amount than 2 µg pLac1Luc) using the calcium phosphate precipitation method. Luciferase activities from cell extracts were measured 32 h after IPTG stimulation and normalized with β-gal activities. The normalized luciferase activities from IPTG-treated cells were compared to those from the corresponding untreated cells, which were considered as 1 (indicated by \*). (B) A stable population of MCF7/LAP5 cells transfected with pEpiLac1Luc was plated at  $1 \times 10^5$  cells per 60 mm plate. One group of cells was continuously maintained in medium containing 1 mM IPTG for indicated times. Another group of cells was incubated in medium containing IPTG for 24 h and washed twice with PBS and cultured in fresh medium without IPTG. At each time point, luciferase activities were measured and normalized with protein concentrations in whole cell extracts. Data were shown as representatives of three independent experiments.

affinity-purified polyclonal antibodies,  $\alpha$ -Nur77 (7),  $\alpha$ -Cyr61 (13) and p27(C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Both luciferase and  $\beta$ -galactosidase ( $\beta$ -gal) activities were measured using reagents from Promega (Madison, WI, USA) according to the manufacturer's instructions.

## RESULTS AND DISCUSSION

### IPTG-Inducible Gene Expression in Human Breast Cancer Cells

We have constructed the episomal vector pEpiLac (Figure 1B; see Materials and Methods), that combines features of the IPTG-inducible expression system and the episomal vector system. Inducible expression of exogenous genes cloned in this vector requires a cellular background that supplies the IPTG-dependent transactivator, LAP-

267 (3). We chose to establish an inducible system in the context of the human breast cancer cell line MCF7 because it is one of the most extensively studied cell lines in cancer biology. MCF7 cells were transfected with pX6LAP267 and pWLneo (5  $\mu$ g and 0.5  $\mu$ g per 100 mm plate, respectively). pX6LAP267 is an autoregulatory vector that expresses LAP267 under an IPTG-inducible promoter, and thus a large amount of LAP267 is produced in the presence of IPTG (Pestov and Lau, unpublished data). pWLneo expresses the neomycin-resistant gene in a constitutive manner (Stratagene). After transfection, cells were selected against G418, and 15 independent, G418-resistant clonal cell lines were isolated.

The efficacy of these clonal cell lines (MCF7-LAP267 cells) to support IPTG-inducible gene expression was evaluated in transient co-transfection assays. These MCF7-LAP267 cells were co-transfected with either of two reporter constructs: pEpiLac1Luc, which expresses the luciferase gene under the IPTG-inducible promoter in the episomal pEpiLac1 vector (Figure 1B); or pLac1Luc, which expresses the luciferase gene under the corresponding promoter in the pLac1 plasmid vector (Figure 1A). These vectors were co-transfected with pPGK $\beta$ Gal, the  $\beta$ -gal expression vector, which serves as an internal control for transfection efficiencies (1). One clonal line, MCF7/LAP5, showed a high level of IPTG-inducible luciferase activity and was further characterized (Figure 2A).

By contrast, the parental MCF7 cells showed nearly undetectable levels of luciferase activity with or without IPTG treatment (data not shown). Where luciferase activity was induced in pLac1Luc up to about 50-fold under the optimal IPTG concentration, the expression of luciferase was inducible up to 300-fold under similar conditions when expressed using the episomal pEpiLac1Luc (Figure 2A). Thus, the episomal vector pEpiLac1 both maintains a low level of basal expression under uninduced conditions and allows a higher level of IPTG-inducible expression when compared to the non-episomal vector pLac1. We have also established the episomal inducible system in the human glioblastoma cell line U-373

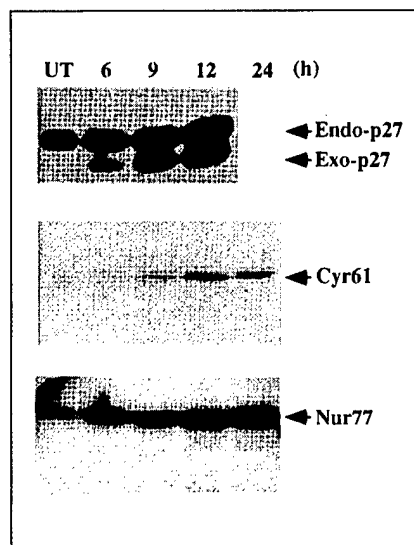
and the human osteogenic sarcoma cell line U2OS, and similar results were obtained (unpublished data).

### On-Off Regulation of Gene Expression

Because expression of exogenous genes in the pEpiLac vector can be turned on efficiently by IPTG induction, we examined whether this expression can be turned off following the removal of the inducer. We generated a population of MCF7/LAP5 cells stably transfected with pEpiLac1Luc and treated these cells with 1 mM IPTG to induce the expression of luciferase (Figure 2B). After 24 h of treatment, the cells were either allowed to continue incubation in the presence of IPTG or washed twice with PBS and cultured in fresh medium without IPTG. After IPTG removal, the luciferase activities remained high for about 6 h and then quickly dropped to near basal levels 24 h thereafter (Figure 2B). However, without removal of IPTG, the luciferase activities continued to accumulate until a plateau was reached (Figure 2B). Thus, expression of the luciferase gene from pEpiLac1 was dependent on the presence of IPTG, and this induction was reversible.

### IPTG-Regulated Expression of Exogenous Genes in Stable Transfectants

Exogenous luciferase reporter gene was efficiently expressed in both transient and stable transfection assays in MCF7/LAP5 cells (Figure 2). Next, we generated populations of MCF7/LAP5 cells stably transfected with pEpiLac vectors expressing either the p27<sup>KIP1</sup> CDK inhibitor (18), the orphan nuclear receptor Nur77 (6) or the angiogenic inducer Cyr61 (2). Cells that survived hygromycin B selection was treated with 1 mM IPTG for various durations. The expression of exogenous gene products was detected in Western blot analysis using affinity-purified polyclonal antibodies (Figure 3). Exogenous proteins was detectable as early as 6 h after IPTG treatment (Figure 3). Overexpression of p27<sup>KIP1</sup> dramatically inhibited cell proliferation rate; cell growth was restored after the removal



**Figure 3. IPTG-induced expression of various exogenous genes in stable transfection assays.** MCF7/LAP5 cells were transfected with 20  $\mu$ g pEpiLac1p27, pEpiLac3Nur77 or pEpiLac3Cyr61 by electroporation. Cells surviving hygromycin B selection were plated in 100 mm dishes ( $1 \times 10^6$  cells each) and stimulated with 1 mM IPTG for indicated times or left untreated (UT). Cells were harvested in TEN buffer, washed twice with cold PBS and lysed in 50  $\mu$ L RIPA buffer. Ten microliters of cell lysates were resolved on 10% SDS-PAGE. The expression of exogenous gene products was detected in Western blot analysis using affinity-purified polyclonal antibodies. Exogenous p27<sup>KIP1</sup> (mouse origin) has a higher electrophoretic mobility than the endogenous human p27<sup>KIP1</sup>, which served as internal control.

of IPTG inducer (unpublished data). This is consistent with the growth inhibitory effect of the p27KIP1 (18). Without IPTG stimulation, exogenous p27KIP1 expression was undetectable (Figure 3), which indicates there was no detectable leakage in this system. Exogenous Cyr61 or Nur77 protein levels in cells not treated with IPTG were similar to the basal level of endogenous proteins (data not shown). The low or undetectable level of leakage in this system allows the regulated expression of growth inhibitory genes such as p27KIP1 and suggests that this system may be useful for cytotoxic genes.

As is the case with other episomal vectors, the pEpiLac DNA can be maintained extra-chromosomally. When cells transfected with the p27KIP1 construct were maintained in media containing hygromycin B for 10 months, episomal DNAs could still be easily harvested using Hirt's extraction (9). After amplification in bacterial cells, the integrity of these episomal DNAs was confirmed by restriction enzyme digestion and PCR analysis (data not shown). Therefore, this system can be applied in genetic selection procedures that require regulated on-off gene expression and recovery of transfected sequences, such as selections using genetic suppressor elements or selectable expression of transient growth arrest phenotype (10,17).

## ACKNOWLEDGMENTS

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## Bop1 Is a Mouse WD40 Repeat Nucleolar Protein Involved in 28S and 5.8S rRNA Processing and 60S Ribosome Biogenesis

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We have identified and characterized a novel mouse protein, Bop1, which contains WD40 repeats and is highly conserved through evolution. *bop1* is ubiquitously expressed in all mouse tissues examined and is upregulated during mid-G<sub>1</sub> in serum-stimulated fibroblasts. Immunofluorescence analysis shows that Bop1 is localized predominantly to the nucleolus. In sucrose density gradients, Bop1 from nuclear extracts cosediments with the 50S-80S ribonucleoprotein particles that contain the 32S rRNA precursor. RNase A treatment disrupts these particles and releases Bop1 into a low-molecular-weight fraction. A mutant form of Bop1, Bop1Δ, which lacks 231 amino acids in the N-terminus, is colocalized with wild-type Bop1 in the nucleolus and in ribonucleoprotein complexes. Expression of Bop1Δ leads to cell growth arrest in the G<sub>1</sub> phase and results in a specific inhibition of the synthesis of the 28S and 5.8S rRNAs without affecting 18S rRNA formation. Pulse-chase analyses show that Bop1Δ expression results in a partial inhibition in the conversion of the 36S to the 32S pre-rRNA and a complete inhibition of the processing of the 32S pre-rRNA to form the mature 28S and 5.8S rRNAs. Concomitant with these defects in rRNA processing, expression of Bop1Δ in mouse cells leads to a deficit in the cytosolic 60S ribosomal subunits. These studies thus identify Bop1 as a novel, nonribosomal mammalian protein that plays a key role in the formation of the mature 28S and 5.8S rRNAs and in the biogenesis of the 60S ribosomal subunit.

Biogenesis of the eukaryotic ribosomes occurs in the nucleolus, a complex nuclear organelle that forms around the nucleolar organizer regions located in heterochromatic chromosomal sites containing multiple rRNA genes (69, 81). The organization of the nucleolus and the assembly of ribosomes are coupled to transcription of rDNA by RNA polymerase I, which synthesizes a large primary precursor transcript. This precursor transcript is then processed into the mature 18S, 5.8S, and 28S/25S rRNAs. These rRNAs are assembled into preribosomes with some 80 ribosomal proteins that are transported into the nucleus, and with the 5S rRNA, transcribed by RNA polymerase III outside of the nucleolus (28). A large number of small nucleolar RNAs (snoRNAs) and nonribosomal proteins are also recruited to the nucleolus to participate in the modification, processing, and assembly of the rRNAs and proteins into ribonucleoprotein (RNP) particles. These preribosomal RNP (pre-rRNP) particles mature into nearly complete ribosomal subunits prior to their export out of the nucleus.

Upon synthesis, the primary precursor rRNA transcript is modified by ribose methylation and pseudouridine conversion and processed through a series of nucleolytic cleavages into the matured rRNAs (21) (see Fig. 6). In vertebrates, the arrangement of the 47S primary precursor transcript begins with a 5' external transcribed spacer (5'-ETS), followed by the 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, internal transcribed spacer 2 (ITS2), 28S rRNA, and the 3' external transcribed spacer (3'-ETS). Although in general, processing events occur in a polar fashion from the 5' to the 3' end of the nascent transcript, differences in the order of processing events and intermediates generated have been reported for different cell types (8, 31, 56). A similar arrangement of the primary

transcript is also found in yeasts (79), and it is generally thought that while specific processing sites might differ between yeasts and vertebrates, parallel processing pathways appear to exist for eukaryotic organisms from yeasts to mammals (75).

A large number of snoRNAs and nonribosomal nucleolar proteins play critical roles in ribosome biogenesis (21, 75). More than 150 snoRNAs have been found in the nucleolus, and some of them play key roles in various pre-rRNA processing events including nucleotide modification and cleavage reactions (45, 76, 83). Although a large number of snoRNAs have been identified and characterized, less is known about the functions of the nonribosomal proteins in the RNP particles, especially in mammalian systems. Many of the recent advances made in the identification of protein factors involved in rRNA processing have come from genetic and biochemical analyses in yeast. Among the yeast proteins known to participate in rRNA processing are endoribonucleases (Rnt1p and RNase MRP) (22, 44), 5'→3' exoribonucleases (Xrn1p and Rat1p) (35, 39), nearly a dozen 3'→5' exoribonucleases that comprise the exosome (1, 48), putative ATP-dependent RNA helicases (Drs1p, Sbp4p, Rrp3p, and Rok1p) (18, 54, 78), and a number of noncatalytic nucleolar proteins (Nop1p, Nop2p, Nopp3p, etc.) (34, 36, 66). The total number of nonribosomal proteins involved in rRNA processing is unknown but is likely to be large, as underscored by the complexity of eukaryotic rRNA modification, processing, and ribosome assembly.

In mammalian systems, relatively few nonribosomal nucleolar proteins have been characterized; the best-studied examples include fibrillarin, a common component of the snoRNPs (43, 53); nucleolin (C23), a pre-rRNA binding protein with multiple functions in processing and ribosome assembly (29, 55, 77); B23, associated with ribosome assembly at later stages of maturation (11); and p120, a nucleolar RNA binding protein that cofractionates with 60S-80S pre-rRNA particles (30, 33). In comparison with yeast, the majority of the molecular players in the mammalian rRNA processing machinery remain un-

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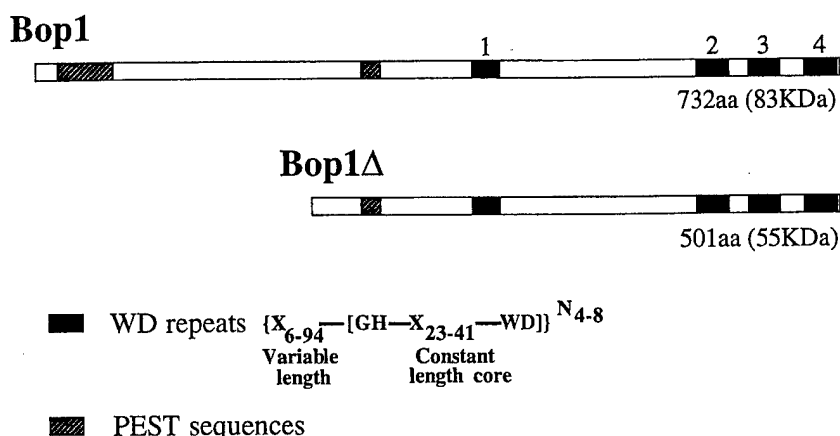


FIG. 1. Structural features of the Bop1 protein. Schematic representation of the full-length murine Bop1 protein (732 aa) and the amino-terminally truncated Bop1Δ (501 aa). The four WD repeats, whose consensus structure is as indicated, are shown as solid boxes. Repeats 1 and 4 are close to the consensus structure, while repeats 2 and 3 are more divergent. PEST sequences, often associated with short-lived regulatory proteins, are shown by hatched boxes.

identified, and their characterization will be necessary to understand fully the mechanism of rRNA processing and ribosomal assembly.

In a previous study, a genetic selection for growth-inhibitory sequences in mouse cells identified a cDNA, *bop1Δ*, whose inducible expression results in a powerful but reversible block in the G<sub>1</sub> phase (58). Further analysis revealed that *bop1Δ* represents a cDNA fragment that encodes an N-terminally truncated form of a novel protein, Bop1. Sequence analysis showed that *bop1* is an evolutionarily conserved gene that encodes an 83-kDa protein with four WD40 repeats (Fig. 1) (58). The WD40 repeat is a sequence motif found in a diverse group of functionally distinct proteins involved in the regulation of myriad cellular processes, including signal transduction, gene transcription, and mRNA modification (51). Moreover, WD40 proteins are often found to form multiprotein complexes, suggesting that the WD40 motifs may be involved in protein-protein interactions (7, 14, 60).

In this study, we demonstrated that Bop1 is a novel component of the 28S branch of the rRNA processing machinery. Bop1 is localized to the nucleolus and forms part of the large RNP particles that probably represent preribosomes. Expression of its N-terminally truncated mutant form, Bop1Δ, specifically inhibits the processing of the 32S precursor to form the mature 5.8S and 28S rRNAs and results in a deficiency of the 60S ribosomal subunits. These results identify Bop1 as a previously unknown player in the processing of the 32S pre-rRNA and in the biogenesis of the 60S ribosomal subunits and suggest that Bop1 may interact with and coordinate the activities of proteins that comprise the pre-rRNA processing machinery.

#### MATERIALS AND METHODS

**Expression plasmids.** An IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression vector, pX11, drives inducible expression with low background in LAP3 cells (59). Expression constructs with cDNAs encoding either Bop1 or Bop1Δ cloned into pX11 have been described previously (58). To express Bop1 or Bop1Δ N-terminally tagged with a hemagglutinin antigen (HA) sequence, a polynucleotide linker encoding the HA tag (23) was inserted into these vectors. To express Bop1 as a glutathione S-transferase (GST) fusion protein in *Escherichia coli*, an *EcoRI* restriction fragment of Bop1 (GenBank accession no. U77415) corresponding to nucleotides (nt) 442 to 2461 was cloned into pGEX-4T-1 (Pharmacia). To express Bop1 in insect cells via a baculovirus expression vector, the full-length 2.4-kb *bop1* cDNA fragment was cloned in pBlueBacHis2A (Invitrogen), thereby driving the expression of a full-length Bop1 protein with an N-terminal 6-histidine tag.

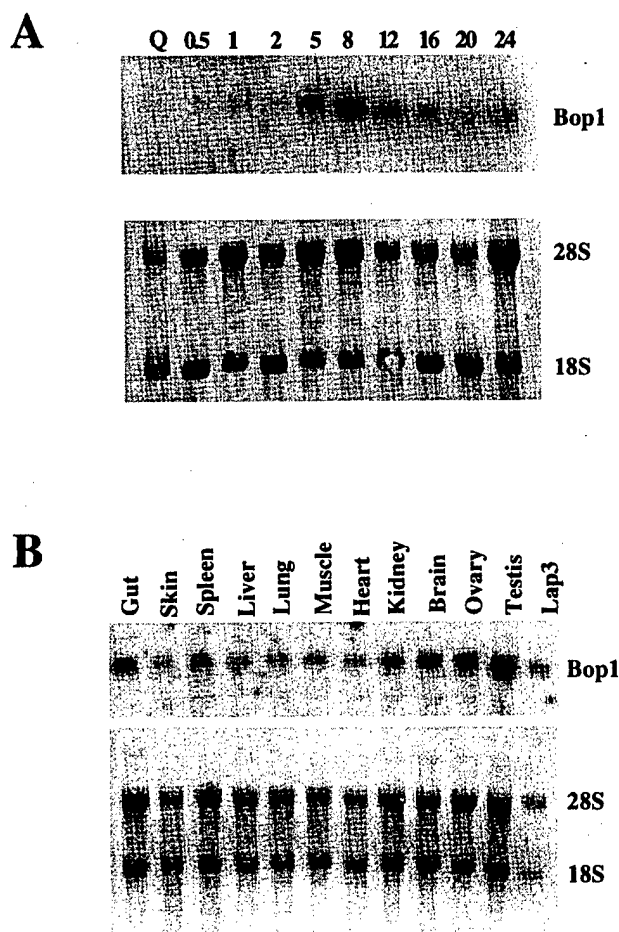


FIG. 2. Expression of *bop1*. (A) BALB/c 3T3 cells were brought to quiescence (Q) by serum starvation and restimulated with 10% fetal bovine serum. RNA was isolated at the indicated times (in hours) after serum stimulation and analyzed by Northern blotting with <sup>32</sup>P-labeled *bop1* cDNA as probe. The RNA blot was stained with methylene blue (lower panel) to show the relative amounts of rRNAs, indicating equal loading of the samples. (B) RNA was isolated from different adult mouse tissues and analyzed by Northern blotting with labeled *bop1* cDNA as probe. The lower panel shows methylene blue staining of the same blot to control for loading of the RNA.



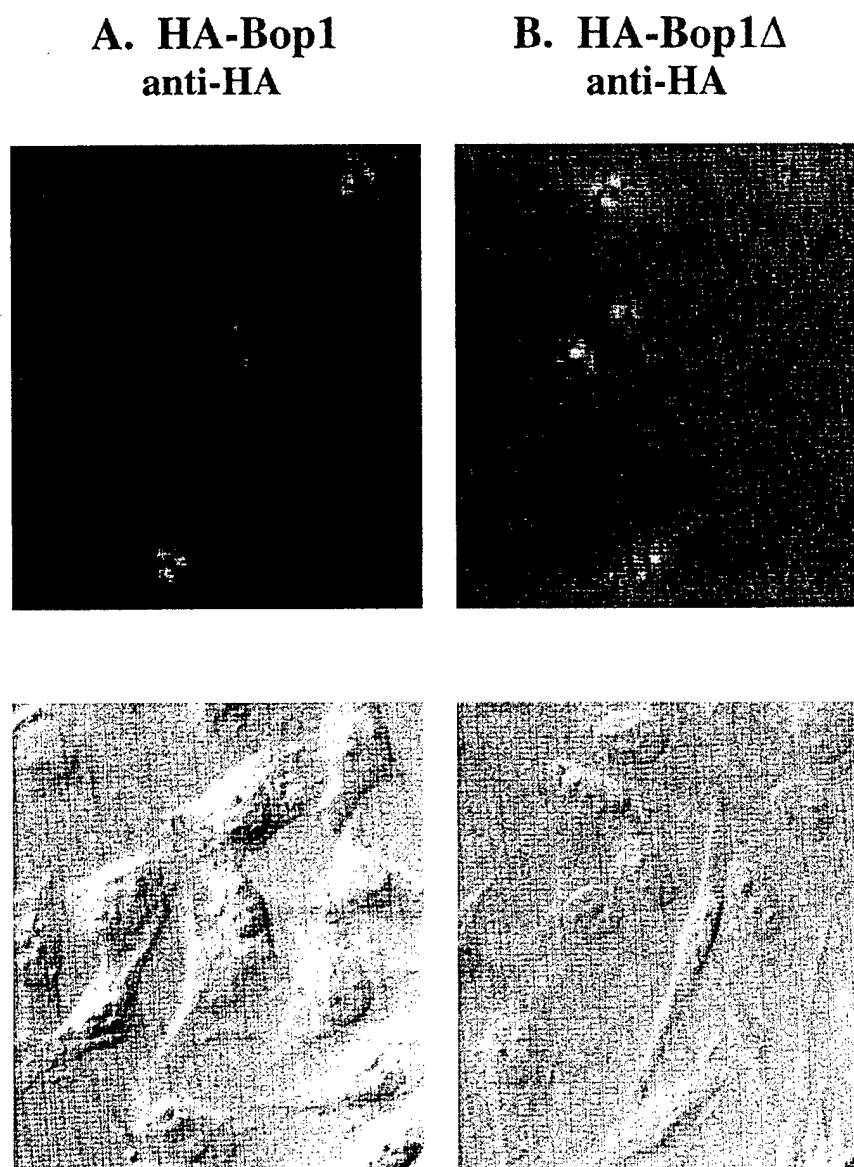


FIG. 3. Subcellular localization of Bop1 by indirect immunofluorescence. (A and B) Pools of LAP3 cells stably transfected with vectors that express HA-tagged Bop1 (A) or Bop1 $\Delta$  (B) were grown on coverslips, induced with IPTG for 12 h, fixed, permeabilized, and stained with monoclonal anti-HA antibody. Antibody-antigen complexes were detected with FITC-conjugated anti-mouse antibody and visualized by fluorescence microscopy. An image of the same field visualized with DIC optics is shown in the lower panels. (C and D) To localize the endogenous Bop1 protein, LAP3 cells were grown on coverslips, fixed, permeabilized, and stained with affinity-purified, polyclonal anti-Bop1 antibodies (C) or the preimmune serum as a control (D).

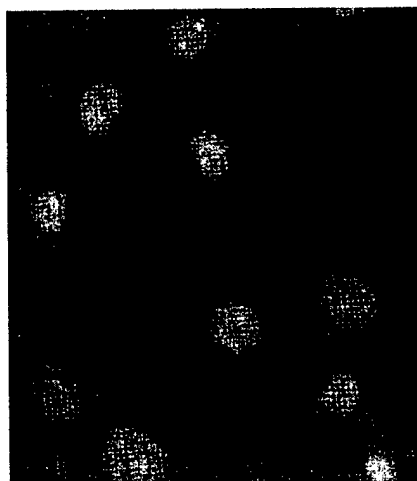
**Cell culture.** BALB/c 3T3 cells were grown in MEM-10 (minimal essential medium with 10% fetal bovine serum). The cells were brought to quiescence by growth to confluence and serum starved in MEM-0.5 for 2 days; serum stimulation was accomplished by changing the medium to MEM-10. LAP3 is a clonal cell line derived from NIH 3T3 cells that constitutively expresses the IPTG-inducible transactivator protein LAP267 (5). To create various clonal cell lines, LAP3 cells were cotransfected with a hygromycin marker plasmid pHyg (73) and either the empty pX11 vector (line 1-1), pX11-Bop1 (line 45), pX11-Bop1 $\Delta$  (lines 6 and 8), pX11-HA-Bop1 (line 10), or pX11-HA-Bop1 $\Delta$  (line 13) using the calcium phosphate coprecipitation method (12). Clonal cell lines were selected following transfection by growth in 130 to 150  $\mu$ g of hygromycin (Boehringer Mannheim) per ml. LAP3-derived cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum (HyClone) and penicillin-streptomycin (Gibco-BRL). IPTG (dioxane free; Sigma) was added to 1 mM where indicated.

**RNA blot analysis.** Where indicated (except for Fig. 11), RNA was isolated using Trizol reagent (Gibco-BRL) as specified by the manufacturer. The samples in the experiment shown in Fig. 11 were withdrawn from sucrose density gradients, treated with proteinase K, and subjected to phenol-chloroform extraction

and isopropanol precipitation. Northern blot hybridization was performed by standard methods (68). The *bop1* cDNA probe was synthesized by random priming in the presence of [ $^{32}$ P]dCTP (Decaprime II kit; Ambion). A 40-mer oligonucleotide (5'-GCGTTCGAAGTGTGATGATCAATGTGTCCTGCAA TTCAC-3') complementary to nt 68 to 108 of the 5.8S rRNA and a 33-mer oligonucleotide (5'-ACTGGTGAGGCAGCGGTCCGGGAGGCGCCGACG-3') complementary to nt 1480 to 1512 of the ITS2 region of the pre-rRNA were 5'-end labeled using [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase.

**Anti-Bop1 antibodies and affinity purification.** To raise polyclonal anti-Bop1 antibodies, a Bop1 polypeptide corresponding to amino acids (aa) 131 to 732 was expressed as a GST fusion protein in *E. coli* (see above). The fusion protein formed insoluble inclusion bodies, which were solubilized in 5 M urea and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6% polyacrylamide). The GST-Bop1 protein band was excised from the gel, and the protein was used for immunization of rabbits at the Immunological Resource Center at University of Illinois at Urbana. Antisera raised against this fusion protein were affinity purified by a standard method (32) by passage through a Bop1-Sepharose column. To prepare the affinity column, full-length Bop1 with an N-terminal histidine tag was expressed in SF9 cells using a bacu-

### C. Lap3 anti-Bop1



### D. Lap3 pre-immune

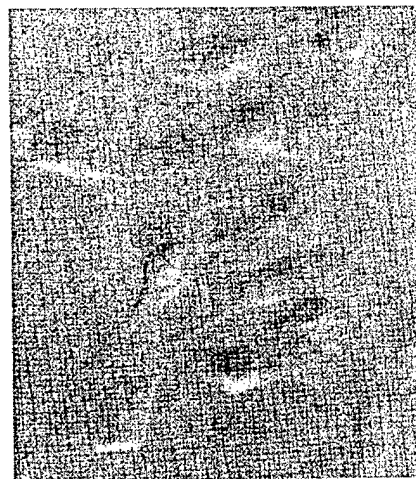


FIG. 3—Continued.

lovirus expression system (Invitrogen) and purified using Pro-Bond resin (Invitrogen) under denaturing conditions as specified by the manufacturer. The protein was further dialyzed against 125 mM sodium phosphate (pH 8)–500 mM NaCl and coupled to cyanogen bromide-activated Sepharose (5 mg of protein per ml of Sepharose slurry) as specified by the manufacturer (Pharmacia). The anti-Bop1 antiserum was diluted 1:10 in phosphate-buffered saline (PBS), passed through the Bop1-Sepharose column, and washed with 10 mM Tris (pH 7.5)–500 mM NaCl. Bound antibodies were first eluted with 100 mM glycine (pH 2.5) and then eluted with 100 mM ethanolamine (pH 11.5). Eluates were combined, dialyzed against PBS, and concentrated with Centricon-30 columns (Amicon) or on a protein A-Sepharose column (68).

**Western blot analysis.** Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (68), and equal amounts of protein, determined by the DC protein assay (Bio-Rad), were resolved by SDS-PAGE. For immunoblot analysis of the sucrose gradient fractions, the proteins from each fraction were precipitated with cold 10% trichloroacetic acid and dissolved in 80  $\mu$ l of Laemmli buffer, half of which was resolved by SDS-PAGE. Western blot analysis was done by standard methods (2) using affinity-purified anti-Bop1 antibodies.

To analyze proteins associated with ribosomal particles in the nucleus and the cytoplasm, cells were lysed as described below for the preparation of nuclear extracts. The cytoplasmic fraction and nuclear sonicate were cleared at  $15,000 \times g$  for 15 min and then centrifuged through 10% sucrose in 10 mM Tris-HCl (pH

7.2)–60 mM KCl–10 mM  $MgSO_4$ –1 mM dithiothreitol (DTT) at  $160,000 \times g$  for 4 h at 5°C. The pellets were dissolved in 1% SDS and used for the isolation of RNA with Trizol and protein analysis by SDS-PAGE. The volumes loaded on a protein gel were normalized for the RNA content of the samples as determined by absorbance at 260 nm and electrophoresis on a formaldehyde-containing agarose gel followed by staining with SYBR Gold (Molecular Probes, Inc.).

**Indirect immunofluorescence.** Cells were grown on coverslips, incubated with IPTG for 12 h, fixed with paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated for 1 h at room temperature with either monoclonal anti-HA antibody (Babco), polyclonal anti-HA antibodies (Babco), affinity-purified anti-Bop1 antibodies, or the anti-fibrillarin monoclonal antibody (72B9) diluted in PBS containing 0.5% bovine serum albumin. After being washed, the cells were incubated with either fluorescein isothiocyanate (FITC)-conjugated or Texas Red-conjugated anti-mouse antibodies (Vector Laboratories) or FITC-conjugated anti-rabbit antibodies (Zymed) and analyzed using a MicroMAX digital camera mounted on a Axioplan II Zeiss microscope with differential interference contrast (DIC) optics.

**Metabolic labeling and analysis of RNA transcripts.** Various cell lines were plated in six-well plates at  $10^5$  cells per well. One day after being plated, the cells were either left untreated or treated with IPTG for 16 h to induce expression. To measure RNA synthesis, the cells were incubated in medium with [ $^3H$ ]uridine (2.5  $\mu$ Ci/ml) for 30 min and then in nonradioactive medium for 2 h. RNA was

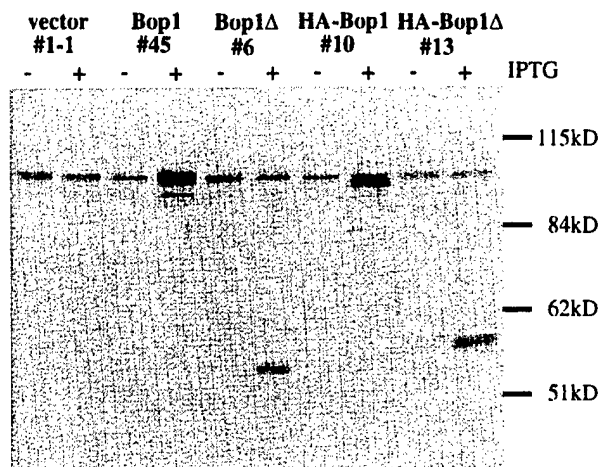


FIG. 4. Characterization of the affinity-purified anti-Bop1 antibodies. Clonal lines of LAP3 cells stably transfected with either the empty vector pX11 (line 1-1), pX11-Bop1 (line 45), pX11-Bop1Δ (line 6), pX11-HA-Bop1 (line 10), or pX11-HA-Bop1Δ (line 13) were either treated with 1 mM IPTG (+) for 12 h or left untreated (-). Cells were lysed in RIPA buffer, and equal amounts of protein from each sample were resolved by SDS-PAGE (8% polyacrylamide) and transferred to a nitrocellulose filter. The blot was incubated with affinity-purified anti-Bop1 antibodies, and chemiluminescent reagents were used for detection. The affinity-purified antibodies recognized a high-molecular-mass band of ~100 kDa (larger than the expected size of 83 kDa), which was increased significantly in a Bop1-overexpressing cell line (line 45). The predicted 55-kDa Bop1Δ fragment was detected in line 6, which inducibly expresses Bop1Δ. Addition of the HA tag caused a minor shift in the mobility of both Bop1 and Bop1Δ in clonal lines 10 and 13, respectively.

isolated using Trizol, and label incorporation was measured by scintillation counting. RNA was subsequently separated on a 1% agarose gel and transferred to a nylon membrane, which was treated with  $\text{En}^3\text{Hance}$  (New England Nuclear) and exposed to film. Pulse-chase experiments were carried out using L-[methyl- $^3\text{H}$ ]methionine due to the rapid turnover of the cellular methionine pool. Cells were preincubated for 15 min in methionine-free medium and then incubated for 30 min in medium containing L-[methyl- $^3\text{H}$ ]methionine (50  $\mu\text{Ci}/\text{ml}$ ). The cells were then chased in nonradioactive medium containing 15  $\mu\text{g}$  of methionine per ml for various times, after which the RNA was isolated using Trizol. RNA from the same number of cells was analyzed as described above. Where indicated, cells were treated with 5  $\mu\text{M}$  5-fluorouridine (FUr) for 15 min prior to labeling. For analysis of the synthesis of 5.8S rRNA,  $^{32}\text{P}$  labeling was used to increase the

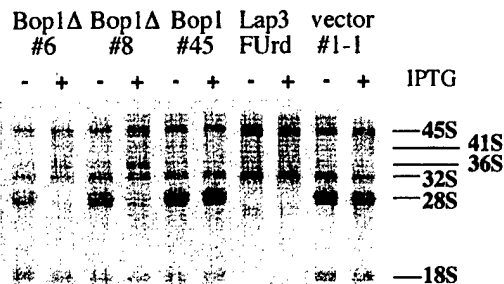


FIG. 5. Expression of Bop1Δ inhibits the generation of 28S rRNA. Clonal cell lines derived from transfection of LAP3 cells with pX11-Bop1Δ (lines 6 and 8), pX11-Bop1 (line 45), or the pX11 vector (line 1-1) were either treated with IPTG (+) for 16 h or left untreated (-). Thereafter, cells were metabolically labeled with [ $^3\text{H}$ ]uridine (2.5  $\mu\text{Ci}/\text{ml}$ ) for 30 min and chased in nonradioactive medium for 2 h. In other samples, LAP3 cells were treated with FUr for 15 min prior to chase. RNA was then isolated, and equal counts per sample were electrophoresed on 1% agarose gel, transferred to a nylon membrane, and visualized by fluorography.

sensitivity of detection. Cells to be labeled with radioactive phosphate were pretreated in phosphate-free medium for 1 h, labeled in medium with [ $^{32}\text{P}$ ]orthophosphate ( $^{32}\text{P}_i$ ) (20  $\mu\text{Ci}/\text{ml}$ ) for 1 h, and chased in nonradioactive medium for 1.5 h. RNA from the same number of cells was separated on a 10% polyacrylamide-7 M urea gel.

**Sucrose density gradient fractionation.** To fractionate cytoplasmic ribosomes, cells were harvested by trypsinization 5 min after the addition of 50  $\mu\text{g}$  of cycloheximide per ml to the medium. Equal numbers of cells (determined with a Coulter counter) from each sample were pelleted by low-speed centrifugation and lysed in 20 mM Tris-HCl (pH 7.2)-130 mM KCl-10 mM  $\text{MgCl}_2$ -2.5 mM DTT-0.5% NP-40-0.5% sodium deoxycholate-10  $\mu\text{g}$  of cycloheximide per ml-0.2 mg of heparin per ml-200 U of RNasin per ml (Promega) for 15 min at 4°C. The lysates were centrifuged at 8,000  $\times g$  for 10 min, and the supernatants were layered on 10 to 45% (wt/wt) sucrose density gradients in 10 mM Tris-HCl (pH 7.2)-60 mM KCl-10 mM  $\text{MgCl}_2$ -1 mM DTT-0.1 mg of heparin per ml. The gradients were centrifuged at 36,000 rpm for 3 h at 5°C in a Beckman SW41Ti rotor and fractionated by upward displacement through a Bio-Rad EM-1 UV monitor for continuous measurement of the absorbance at 254 nm. Sedimentation constants were calculated as described previously (46).

To fractionate nuclear extracts, cells were washed twice in PBS and once in ice-cold hypotonic wash buffer (10 mM Tris [pH 7.4], 10 mM KCl, 2 mM  $\text{MgCl}_2$ ) and then left to swell for 20 min in hypotonic lysis buffer (10 mM Tris [pH 7.4], 10 mM KCl, 2 mM  $\text{MgCl}_2$ , 0.05% Triton X-100, 1 mM EGTA, 1 mM DTT, 40  $\mu\text{g}$  of phenylmethylsulfonyl fluoride per ml, 10  $\mu\text{l}$  of protease inhibitor cocktail [Sigma] per ml) (40). The cells were forced through a 25-gauge needle and centrifuged for 5 min at 700  $\times g$  to obtain a pellet of nuclei, which was resuspended in 25 mM Tris (pH 7.5)-100 mM KCl-1 mM DTT-2 mM EDTA-0.05% NP-40-1 mM NaF-40  $\mu\text{g}$  of phenylmethylsulfonyl fluoride per ml-10  $\mu\text{l}$  of protease inhibitor cocktail per ml-0.1 U of RNasin (Promega) per ml and sonicated four times for 15 s each with a microtip (Heat Systems Ultrasonics, Inc.). The nuclear lysate was centrifuged at 15,000  $\times g$  for 15 min, and the resulting supernatant was overlaid on 10 to 30% (wt/wt) sucrose gradients in 25 mM Tris (pH 7.5)-100 mM KCl-1 mM DTT-2 mM EDTA and centrifuged at 36,000 rpm for 3 h at 5°C in a Beckman SW41Ti rotor. Where indicated, the nuclear extract was treated with RNase A (100  $\mu\text{g}/\text{ml}$ ) for 10 min at 30°C before being loaded on the gradient. The gradients were analyzed as above.

## RESULTS

**Expression of the *bop1* gene.** Expression of an N-terminally truncated form of Bop1, Bop1Δ, results in a strong but reversible  $G_1$  growth arrest (58). This observation raised the possibility that the activity of Bop1 might affect cell cycle progression and that expression of the *bop1* gene might be linked to the proliferative state. To determine if *bop1* expression is growth regulated, we analyzed RNA isolated from BALB/c 3T3 cells stimulated to reenter the cell cycle from quiescence (Fig. 2A). *bop1* expression was low in quiescent cells and was induced after serum stimulation, starting at 5 h, reaching maximal level at 8 h, and slowly declining thereafter. Thus, *bop1* expression increases in cells stimulated to proliferate, with maximal expression in mid- $G_1$ . A survey of RNA isolated from various mouse tissues showed the presence of the 2.6-kb *bop1* mRNA in all tissues tested, with the highest level in the testis (Fig. 2B).

**Bop1 and Bop1Δ are localized in the nucleolus.** In an effort to determine the function of Bop1, we ascertained its subcellular localization. First, we prepared IPTG-inducible expression constructs that drive the expression of either HA-tagged Bop1 or Bop1Δ in LAP3 cells, an NIH 3T3-derived cell line that supports IPTG-regulated gene expression (see Materials and Methods). Stable pools of transfected LAP3 cells were induced with IPTG and subjected to immunofluorescence analysis using monoclonal anti-HA antibody (Fig. 3A and B). Cells that express either HA-tagged Bop1 or HA-tagged Bop1Δ showed strong antibody staining in the nucleoli, although a weak and diffuse nucleoplasmic staining was also observed. Examination of the same fields using DIC optics confirmed the nucleolar localization. Double staining with polyclonal anti-HA antibodies and a monoclonal antifibrillarin antibody (72B9) (62) also confirmed the nucleolar localization of the HA-tagged Bop1 and Bop1Δ (data not shown).

To reinforce and substantiate the above findings, we deter-

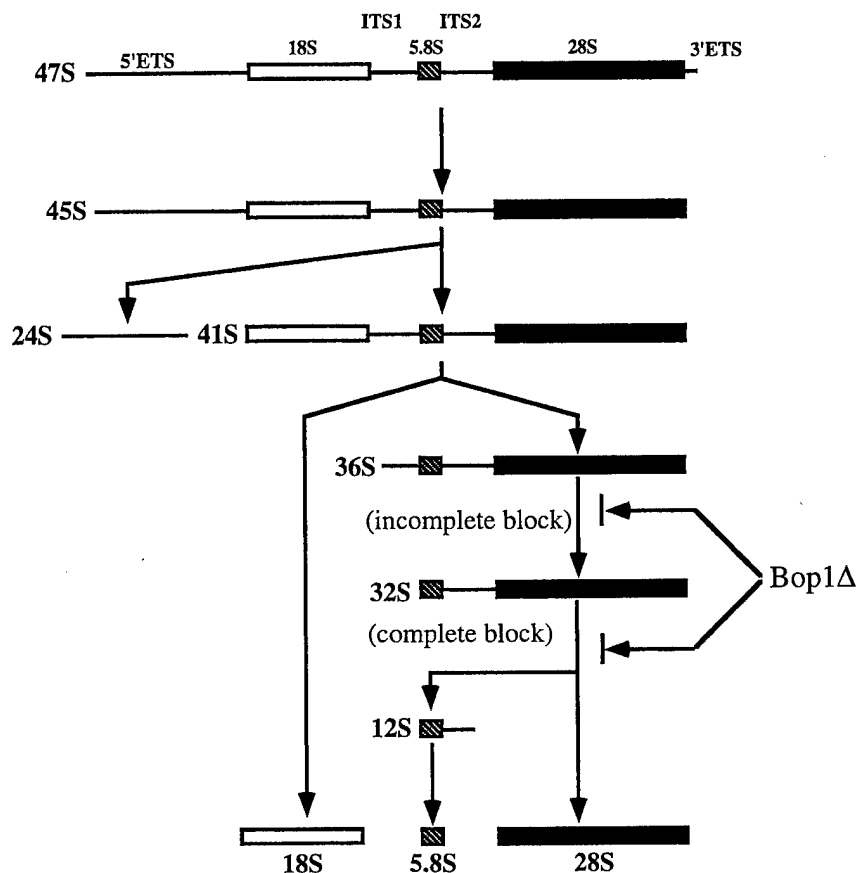


FIG. 6. Schematic representation of the mammalian rRNA-processing pathway. Mammalian rRNA is transcribed as a single precursor which is further processed by successive nucleolytic cleavages that lead to elimination of the external transcribed spacers, 5'ETS and 3' ETS, and the internal transcribed spacers, ITS1 and ITS2. The sedimentation coefficients (S) of various intermediates and mature products of the processing pathway are indicated. The mammalian 47S precursor is rapidly cleaved at the 5'ETS and at the 3'ETS to give rise to the 45S pre-rRNA. Further processing at the 5'ETS takes place, giving rise to a 41S rRNA precursor, which is rapidly processed to the 18S rRNA and a 36S precursor RNA that contains the sequences of the 5.8S and 28S rRNAs with an intervening sequence (ITS2). The 36S precursor then undergoes cleavage at the 5' end to give rise to a 32S precursor, which is processed to the 28S rRNA and a 12S RNA; the 12S RNA is then further processed to form the 5.8S rRNA.

mined the subcellular localization of the endogenous Bop1 protein by using affinity-purified anti-Bop1 antibodies (see Materials and Methods). These antibodies reacted specifically with Bop1 in cell lysates on a denaturing Western blot, where Bop1 exhibited an apparent molecular mass of ~100 kDa (larger than the calculated size of 83 kDa) (Fig. 4). These antibodies were used to demonstrate that various IPTG-inducible cell lines express the appropriate gene products upon induction (Fig. 4). Using these antibodies, endogenous Bop1 was also localized to the nucleolus by immunofluorescence analysis whereas preimmune serum from the same rabbit did not show any staining (Fig. 3C and D). No anti-Bop1 staining was detected in the cytoplasm, although a low level of diffuse staining was also detected in the nucleoplasm. Together, these results show that both endogenous Bop1 and exogenously expressed Bop1 or Bop1Δ are localized predominantly in the nucleolus.

**Involvement of Bop1 in rRNA processing.** The localization of Bop1 in the nucleolus—the site of rRNA synthesis, modification, processing, and ribosome biogenesis—suggests that it may play a role in one or more of these processes. That Bop1Δ is colocalized with Bop1 and its expression inhibits cell growth suggests that it might interfere with the normal function of Bop1, most probably acting as a dominant negative mutant.

To investigate the function of Bop1, we first examined whether expression of Bop1 or Bop1Δ affects rRNA synthesis. For this purpose, we created a set of cell lines that express Bop1 or Bop1Δ in an IPTG-inducible manner (see Materials and Methods). These inducible cell lines were either untreated or treated with IPTG, metabolically labeled with [<sup>3</sup>H]uridine for 30 min, and subjected to a 2-h chase. Inducible overexpression of Bop1 (line 45) showed no effect on the synthesis of the large rRNA precursors (45S and 32S) or the mature 18S and 28S rRNAs, resulting in an rRNA pattern indistinguishable from that of the untreated cells or the control cell line (line 1-1; the parental LAP3 cells transfected with the empty cloning vector) (Fig. 5). By contrast, expression of Bop1Δ (lines 6 and 8) strongly inhibited production of the 28S rRNA whereas the levels of 18S rRNA and the 45S precursor were unchanged (Fig. 5). Bop1Δ expression also resulted in accumulation of the 36S rRNA, normally present in small amounts. In control experiments where LAP3 cells were treated with FUr, a known inhibitor of rRNA processing (84), a complete blockade of production of both 28S and 18S rRNA was observed without affecting the formation of the 45S and 32S precursors (Fig. 5). Together, these results suggested that Bop1Δ did not significantly affect the synthesis and initial processing of the primary

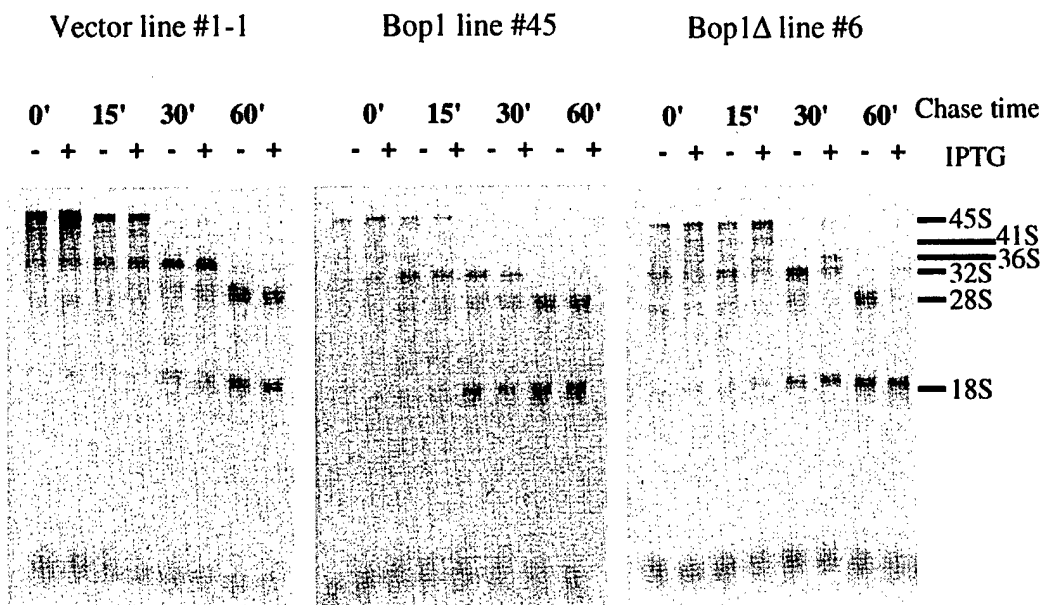


FIG. 7. Bop1Δ inhibits processing of the 36S and 32S precursors to form the 28S rRNA. Clonal LAP3 cell lines transfected with either the empty pX11 vector (line 1-1), pX11-Bop1 (line 45), or pX11-Bop1Δ (line 6) were either left untreated (–) or treated with 1 mM IPTG for 16 h (+) and pulse-labeled with  $^3\text{H}$ -labeled methyl methionine for 30 min. After a chase in nonradioactive medium plus excess methionine for the indicated times, RNA was isolated, resolved on a 1% agarose gel, transferred to a membrane, and visualized by fluorography.

rRNA transcript but specifically compromised later processing steps that lead to maturation of the 28S rRNA.

In mammalian cells, the 36S precursor is processed to form the 32S pre-rRNA, which is further processed to produce the

mature 28S rRNA and a 12S precursor from which the 5.8S rRNA is generated (Fig. 6). The concomitant block of the 28S rRNA and accumulation of the 36S pre-rRNA described above suggested that Bop1Δ may inhibit the processing steps that

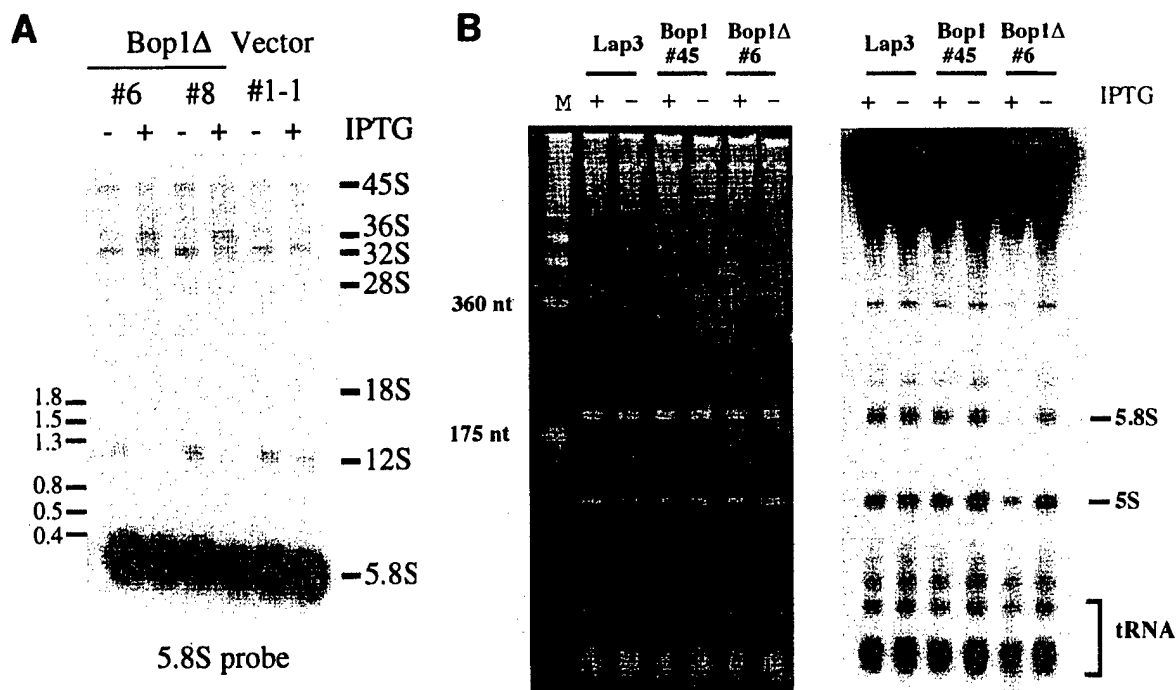


FIG. 8. Bop1Δ expression inhibits generation of the 12S precursor and the 5.8S rRNA. (A) Clonal LAP3 cell lines transfected with either the empty pX11 vector or pX11-Bop1Δ (lines 6 and 8) were either left untreated or treated with IPTG for 16 h. RNA isolated from the same number of cells was separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with an oligonucleotide probe complementary to a region in 5.8S rRNA. (B) The parental LAP3 cells or clonal cell lines transfected with either pX11-Bop1 (line 45) or pX11-Bop1Δ (line 6) were either left untreated or treated with IPTG for 16 h and metabolically labeled with  $^{32}\text{P}_i$  (20  $\mu\text{Ci}/\text{ml}$ ) for 1 h. Following a chase in nonradioactive medium for 1.5 h, RNA was isolated, and equal amounts of RNA from each sample were resolved on a 10% denaturing polyacrylamide gel, which was stained with ethidium bromide for photography (left panel) and dried for autoradiography (right panel).

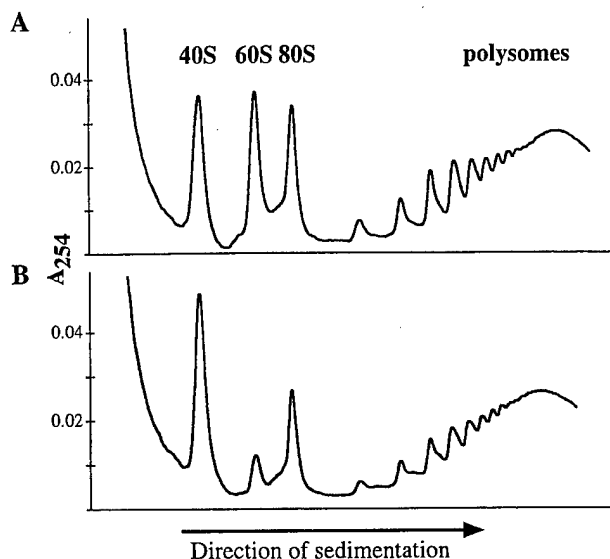


FIG. 9. Expression of Bop1 $\Delta$  causes a deficit in 60S ribosomal subunits. The inducible Bop1 $\Delta$  expression cell line, line 6, was grown in the absence (A) or presence (B) of IPTG as indicated for 38 h. Cytoplasmic extracts were isolated and separated on a 10 to 45% sucrose density gradient. Profiles of absorbance at 254 nm ( $A_{254}$ ) profiles revealed the positions and relative amounts of the ribosomal subunits in the gradient.

convert the 36S pre-rRNA into the mature 28S rRNA. To evaluate this interpretation and to identify the specific rRNA processing blocks due to Bop1 $\Delta$  expression, pulse-chase experiments were performed by metabolic labeling with  $^3\text{H}$ -labeled methyl methionine. LAP3-derived cell lines that either inducibly express Bop1 (line 45) or Bop1 $\Delta$  (line 6) and the control cell line transfected with empty vector (line 1-1) were either left untreated or treated with IPTG, pulse-labeled for 30 min, and chased with excess nonradioactive methionine for various durations (Fig. 7). In the absence of IPTG, each cell line showed the synthesis of the 45S precursor, which was rapidly converted to the 32S pre-rRNA and the mature 18S rRNA within 30 min after pulse-labeling. The 36S pre-rRNA is relatively short-lived and does not accumulate significantly under these conditions. Processing of the 32S pre-rRNA to the mature 28S rRNA appeared to be complete by 60 min after pulse-labeling. The addition of IPTG to the control cell line (line 1-1) or to the Bop1-expressing cell line (line 45) did not show any effect on rRNA synthesis or processing. By contrast, expression of Bop1 $\Delta$  (line 6) resulted in accumulation of the 36S pre-rRNA and diminution of the amount of 32S pre-rRNA. Whereas production of the 18S rRNA appeared normal, maturation of the 28S rRNA was completely blocked. By 60 min after pulse-labeling, both the 36S and 32S pre-rRNAs appeared degraded rather than processed, resulting in the presence of only the 18S rRNA. These results show that expression of Bop1 $\Delta$  does not affect production of the 18S rRNA but completely inhibits formation of the 28S rRNA. Moreover, Bop1 $\Delta$  expression elicits an incomplete block in the conversion of the 36S to the 32S pre-rRNA and a complete block in the processing of the 32S to the 28S rRNA (Fig. 6 and 7).

**Bop1 $\Delta$  expression inhibits de novo generation of the 12S and 5.8S rRNAs.** The 32S pre-rRNA is normally processed to form the mature 28S rRNA and the 12S precursor, from which the 5.8S rRNA is generated (Fig. 6) (21). The complete inhibition of 28S rRNA generation by Bop1 $\Delta$  suggested that formation of the 12S and 5.8S rRNAs might also be inhibited. To

test this possibility, we examined the levels of the 12S pre-rRNA and the 5.8S rRNA in Bop1 $\Delta$ -expressing cells. Total RNA was isolated from various cell lines grown in the presence or absence of IPTG and subjected to RNA blot analysis using the coding region of the 5.8S rRNA as probe (Fig. 8A). This probe should hybridize to and reveal the steady-state levels of the 45S, 41S, 36S, 32S, and 12S precursors, as well as the mature 5.8S rRNA (Fig. 6). These experiments showed that expression of Bop1 $\Delta$  (lines 6 and 8) resulted in the diminution of the amount of 12S pre-rRNA to an undetectable level, with a concomitant accumulation of the 36S precursor. The steady-state level of the mature 5.8S rRNA appeared unchanged during the course of this experiment, given the high levels of preexisting rRNAs.

Inhibition of 12S pre-rRNA formation predicts an inability to generate mature 5.8S rRNA from the newly synthesized rRNA transcript. To test this possibility, we carried out a pulse-labeling experiment to examine the formation of the 5.8S rRNA. Various cell lines grown with or without IPTG were pulse-labeled with  $^{32}\text{P}_i$  for 1 h and then chased in nonradioactive medium for 1.5 h, and total RNA isolated from these cells was resolved by denaturing acrylamide gel electrophoresis (Fig. 8B). Whereas overexpression of Bop1 had no effect (line 45), expression of Bop1 $\Delta$  (line 6) resulted in nearly complete inhibition in 5.8S rRNA synthesis (Fig. 8B). These results show that expression of Bop1 $\Delta$  leads to a block in the processing of the 32S pre-rRNA into the 28S rRNA as well as into the 12S and 5.8S rRNAs. Interestingly, a small decrease in the amount of 5S rRNA was also observed, possibly reflecting a coordinate regulation between pre-rRNA processing and 5S rRNA transcription (37). In addition, the recruitment of 5S rRNA to the pre-60S particle was shown to be necessary for efficient processing of the 27S rRNA precursor in yeast (16). It is possible to speculate that the recruited 5S rRNA might be degraded together with the improperly processed 32S rRNA.

**Expression of Bop1 $\Delta$  causes a deficit of the 60S ribosomal subunit.** Since both 28S and 5.8S rRNAs are incorporated into the 60S ribosome, a specific block in their generation may result in a deficit in ribosome biogenesis. To examine this possibility, we used sucrose density gradient centrifugation to fractionate cytoplasmic extracts of a cell line that inducibly expressed Bop1 $\Delta$  (line 6) (Fig. 9). Upon Bop1 $\Delta$  expression by IPTG treatment, significantly fewer free 60S ribosomal subunits were observed in the cytoplasm whereas the 40S ribosomal subunits were accumulated to a higher level. Compared to untreated cells, the amount of 80S ribosomes was also decreased. The simplest interpretation of these results is that the 60S ribosomal subunits cannot form due to the block in 28S and 5.8S rRNAs synthesis and that the 40S ribosomal subunits accumulate due to a stoichiometric imbalance with the 60S subunit. Although the peaks that corresponded to polysomes appeared somewhat diminished by Bop1 $\Delta$  expression, no global polysome disassembly was detected. This is consistent with the observation that the overall rate of translation was unaffected by Bop1 $\Delta$  expression within the duration of these experiments as judged by metabolic labeling with [ $^{35}\text{S}$ ]methionine (data not shown).

**Bop1 and Bop1 $\Delta$  are cosedimented with the 50S-80S pre-rRNP particles.** The processing of eukaryotic rRNA occurs coordinately with the assembly of ribosomal particles in the nucleolus (81, 85). Pre-rRNP particles have been identified as several complexes by sucrose density gradients. In the presence of EDTA, these complexes, which contain the 45S, 36S, and 32S pre-rRNAs, sediment in the range of 50S to 80S (42, 47, 82). The apparent involvement of Bop1 in pre-rRNA processing suggested that Bop1 might be associated with the pre-

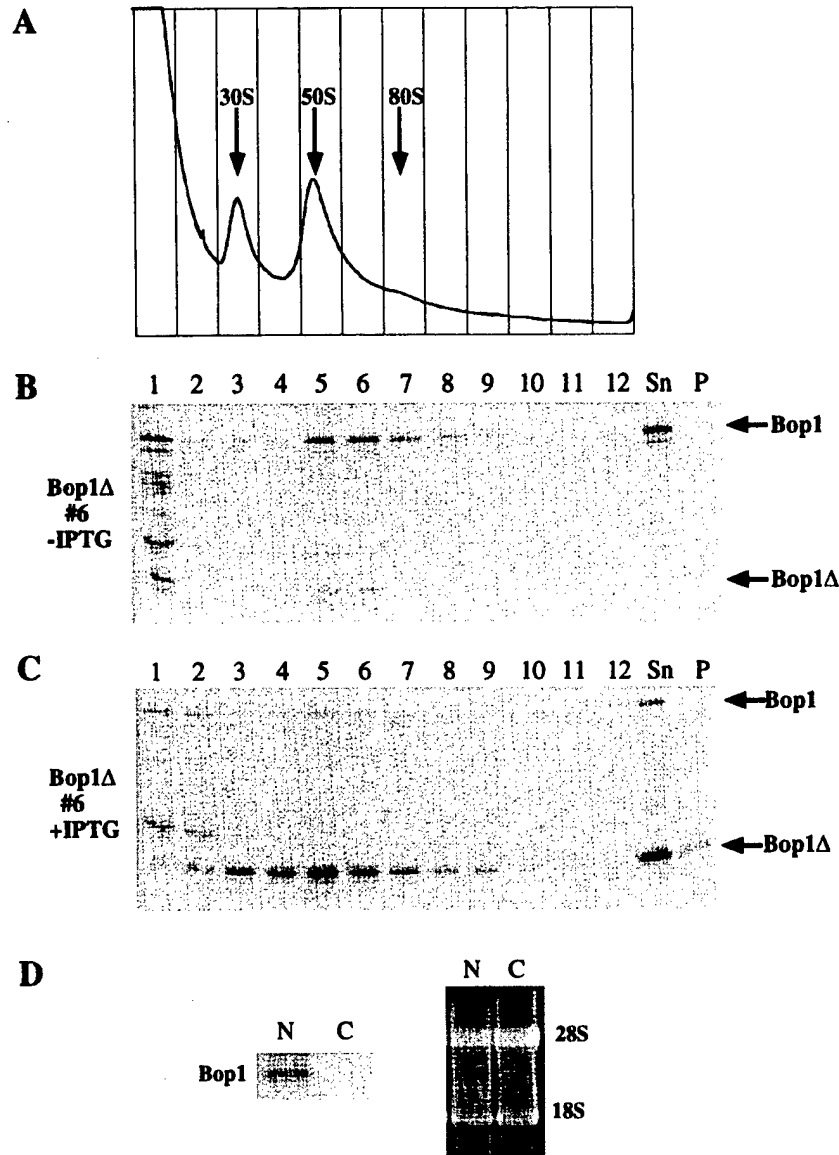


FIG. 10. Bop1 and Bop1Δ cofractionate with the 50S-80S pre-rNP particles in nuclear extracts. (A to C) The inducible Bop1Δ expression cell line (line 6) was grown in the presence (C) or absence (A and B) of IPTG for 24 h as indicated. Nuclear extracts isolated from these cells were analyzed on 10 to 30% sucrose density gradients, which were fractionated with continuous monitoring of absorbance at 254 nm (A). Individual fractions were electrophoresed on an SDS-10% polyacrylamide gel and subjected to immunoblotting analysis using affinity-purified anti-Bop1 antibodies. Sn, unfractionated soluble nuclear extracts; P, insoluble pellet. (D) (Left) Immunoblot analysis with anti-Bop1 antibodies detects Bop1 in nuclear RNPs (N) but not cytoplasmic ribosomes (C). (Right) Electrophoretic analysis of RNA in the fractions used for immunoblotting. RNA was extracted from the nucleoprotein complexes and separated by electrophoresis on a formaldehyde-containing agarose gel to demonstrate the presence equivalent amounts of rRNA in both samples.

rRNP particles. To investigate this possibility, nuclear extracts prepared from the inducible Bop1Δ-expressing cell line (line 6) were analyzed by sucrose gradient centrifugation (Fig. 10A). Proteins from each gradient fraction were assayed for endogenous Bop1 and ectopically expressed Bop1Δ by Western blot analysis. When cells were grown in the absence of IPTG, endogenous Bop1 protein sedimented primarily in fractions 5 to 7, which corresponded to the 50S-80S particles (Fig. 10B and C). When expressed, Bop1Δ was distributed more broadly from fractions 3 through 7 with a peak at fraction 5, where the peak of the endogenous Bop1 was also found. Thus, both Bop1 and Bop1Δ were found in large nuclear particles with sizes similar to pre-rNP particles. However, Bop1 is not a component of matured ribosomes, since Western blotting of a cyto-

plasmic ribosomal preparation did not detect any Bop1 protein (Fig. 10D).

To substantiate the observation that Bop1 cosediments with the 50S-80S preribosomes, nuclear extracts were fractionated by sucrose density gradients and proteins and RNA from each gradient fractions were analyzed in parallel (Fig. 11). Proteins were resolved by electrophoresis, blotted, and probed with anti-Bop1 antibodies, while RNA from the same samples was electrophoresed, blotted, stained with methylene blue, and probed with the <sup>32</sup>P-labeled DNA fragment from the ITS2 region, which can recognize the 47S, 45S, 41S, 36S, and 32S pre-rRNAs. This analysis showed that the Bop1 protein cosedimented with rRNP particles that contained the 32S precursor RNA, found in fractions 5 to 8, and that the peaks for

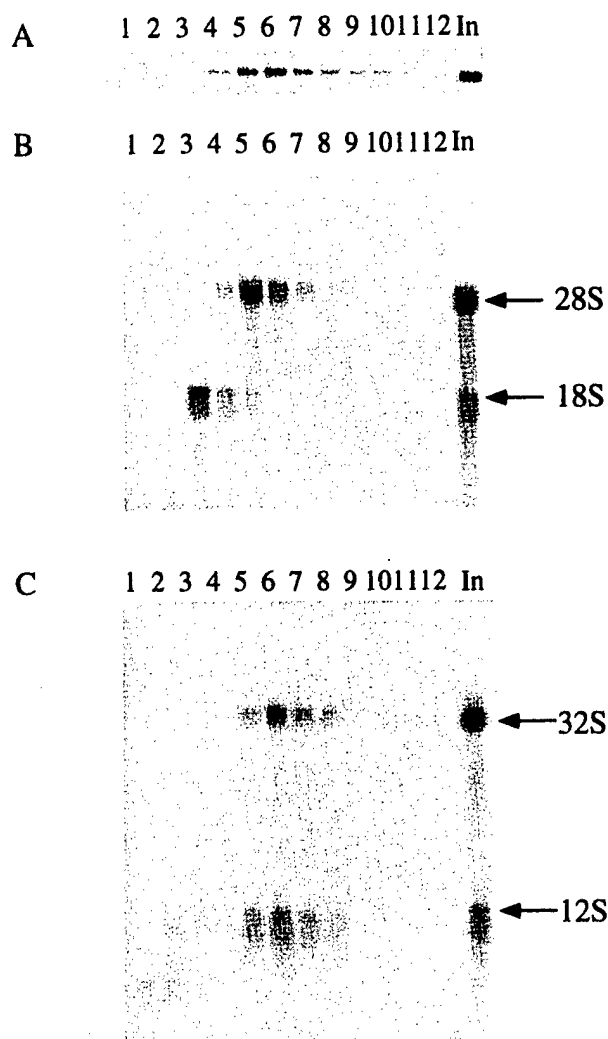


FIG. 11. Bop1 cofractionates with the 32S precursor and 28S rRNA in the nuclear extract. Nuclear extracts from LAP3 cells were isolated and separated on a 10 to 30% sucrose density gradient. The fractions collected were subjected to parallel analysis of protein and RNA. (A) Proteins from various fractions were electrophoresed on an SDS-10% polyacrylamide gel and immunoblotted with affinity-purified anti-Bop1 antibodies. (B) RNAs from each fraction analyzed in panel A were resolved on a 1% agarose gel and transferred to a nylon filter, which was stained with methylene blue. The staining pattern reveals the fractions containing the 18S and 28S rRNAs. (C) The nylon filter shown in panel B was subjected to hybridization using radioactively labeled sequences from ITS2 as a probe, revealing the 32S precursor RNA. In, unfractionated soluble nuclear extract.

both Bop1 and the 32S rRNA both occurred in fraction 6. The 18S rRNA was found mainly in fractions 3 and 4, whereas the 28S rRNA was found in fractions 4 to 7, with the peak in fraction 5. Thus, these results show that Bop1 cosediments with the rRNP particles containing the 32S pre-rRNA, consistent with a role for Bop1 in the processing of the 32S pre-rRNA.

To confirm that Bop1 is a component of rRNP particles, nuclear extracts were either untreated or treated with RNase A before being subjected to fractionation on a sucrose gradient (Fig. 12). As expected, Bop1 cofractionated with rRNP particles in the absence of RNase A. Treatment with RNase completely destroyed the rRNP particles, as shown by the absorbance of the gradient at 254 nm. Bop1 protein was completely shifted to the top of the gradient on nuclease digestion, con-

sistent with its release from rRNP particles. In separate experiments, Bop1 $\Delta$  was also released from the 50S-80S fractions to the top of the gradient on RNase A treatment (data not shown). Together, these results show that as a nucleolar protein, Bop1 is a component of the RNP particles that cosediment with the pre-rRNP particles containing the 32S pre-rRNA.

## DISCUSSION

In this study we identified Bop1 as a novel participant in the mammalian nucleolar rRNA processing and ribosome biogenesis machinery. This conclusion is drawn based on data accumulated through two approaches: (i) biochemical studies show that Bop1 is a nucleolar protein that forms part of a large, RNA-containing protein complex that cosediments with pre-rRNP particles containing the 32S pre-rRNA; and (ii) functional analyses indicate that Bop1 plays a role in the maturation of the 28S and 5.8S rRNAs and the biogenesis of the 60S ribosomal subunit.

Several lines of evidence provide support for the conclusion that Bop1 is a nonribosomal nucleolar protein that constitutes a component of pre-rRNP particles. Immunofluorescence analysis demonstrates that both endogenous and ectopically expressed Bop1 are localized to the nucleolus (Fig. 3). Sucrose density gradient fractionation of nuclear extracts shows that Bop1 forms part of large ribonucleoprotein complexes that sediment at 50S-80S (Fig. 10). Treatment of the nuclear preparations with RNase A, which destroys pre-rRNP particles, releases Bop1 into low-molecular-weight fractions at the top of the gradient (Fig. 12). Thus, while Bop1 is not part of the mature cytosolic ribosomes, these data strongly indicate that it is a component of the pre-rRNP particles.

To analyze the function of Bop1, we took advantage of an N-terminally truncated derivative, Bop1 $\Delta$ , as a means of interfering with the activity of the wild-type protein in a dominant manner. Bop1 $\Delta$  displays the same nucleolar localization as Bop1 and a similar sedimentation profile in a sucrose density gradient, indicating that it resides in the same RNP complexes as the wild-type protein and thus retains a subset of its functions. Since Bop1 $\Delta$  retains the WD40 motifs present in the full-length Bop1 (Fig. 1), it is plausible to speculate that Bop1 $\Delta$  is able to interact with many of the same proteins with which Bop1 interacts. However, Bop1 $\Delta$  apparently interferes with the normal function of Bop1. Indeed, expression of Bop1 $\Delta$  results in a serious defect in the processing of the 28S and 5.8S rRNAs and a deficit of mature 60S ribosome subunits. This conclusion is supported by a preponderance of evidence. (i) Metabolic labeling shows that expression of Bop1 $\Delta$  leads to a specific inhibition of 28S and 5.8S rRNA maturation while having no effect on the 18S rRNA (Fig. 5 and 8). (ii) Pulse-chase analysis reveals a partial processing block in the conversion of the 36S to the 32S pre-rRNA and a complete block in the processing of the 32S pre-rRNA to the mature 28S rRNA and 12S pre-rRNA and consequently to the 5.8S rRNA (Fig. 7 and 8). (iii) Examination of the steady-state RNA levels shows an accumulation of the 36S rRNA and an inhibition of 12S pre-rRNA (Fig. 8A), confirming the results of pulse-chase labeling. (iv) Sedimentation gradients show a marked decrease in the number of cytosolic 60S ribosomal subunits (Fig. 9). These findings strongly implicate Bop1 as an important player in rRNA maturation, specifically in the conversion of the 32S pre-rRNA into the 28S rRNA and the 12S precursor. Moreover, both Bop1 and Bop1 $\Delta$  cosediment with pre-rRNPs that contain the 32S rRNA precursors (Fig. 10 and 11), further corroborating



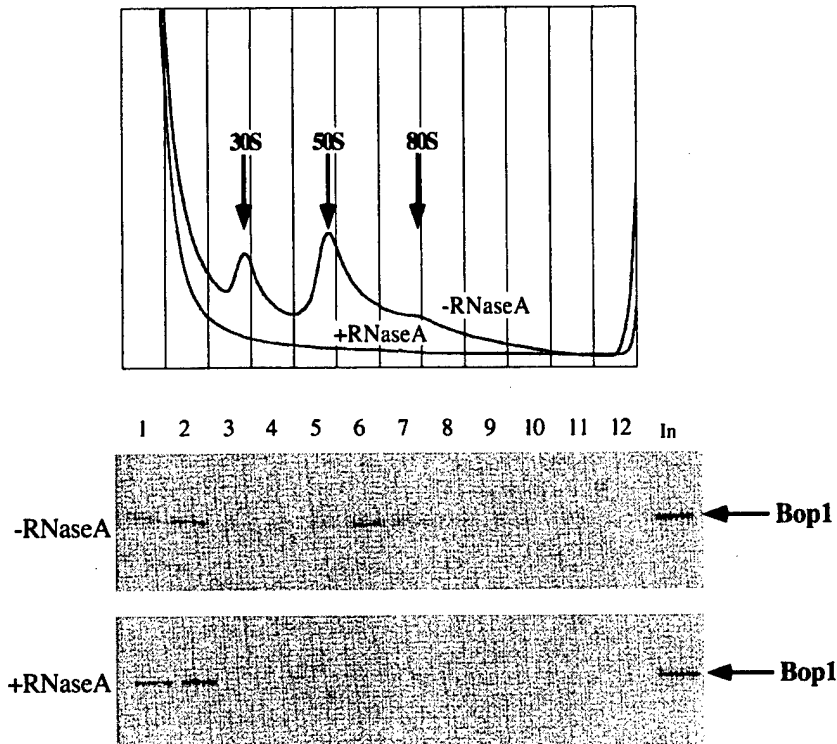


FIG. 12. Bop1 is part of an RNP complex. Nuclear extracts from LAP3 cells were either treated with RNaseA or left untreated as indicated and analyzed on 10 to 30% sucrose density gradients. The gradients were fractionated and monitored for absorbance at 254 nm (top panel). Various fractions were subjected to SDS-PAGE followed by immunoblotting using affinity-purified anti-Bop1 antibodies. In, unfractionated soluble nuclear extract.

the conclusion that Bop1 is involved in the maturation of the 5.8S and 28S rRNAs, a process with which Bop1 $\Delta$  interferes.

Expression of Bop1 $\Delta$  under the control of an inducible promoter was previously shown to cause a powerful but reversible G<sub>1</sub> growth arrest in mouse fibroblasts (58). The precise mechanism by which Bop1 $\Delta$  causes this growth inhibition is unclear. Nevertheless, it is possible to envisage at least two distinct mechanisms by which Bop1 $\Delta$  may affect cell cycle progression. First, growth arrest due to Bop1 $\Delta$  may be a secondary effect resulting from perturbations in the translation machinery caused by deficiency of 60S ribosomal subunits. For example, deficiencies in the translation initiation factor eIF4E/CDC33 lead to a G<sub>1</sub> growth arrest in yeast (9). Alternatively, Bop1 might play a dual role in both pre-rRNA processing and cell cycle progression, thereby mediating a cross talk between these two distinct cellular pathways. The nucleolus has been implicated in functions other than rRNA processing and ribosome biogenesis (57), including the regulation of cellular exit from mitosis (4). Exosomes and Xrn1p exonuclease function in mRNA in addition to rRNA processing (10, 17, 50) and may thus affect the synthesis of cell cycle regulators. The possibility that Bop1 might exert an effect on the cell cycle machinery hints at an as yet poorly understood link between the cellular capacity for coordinating protein synthesis and cell cycle progression. Understanding the precise role of Bop1 in cell cycle progression will require further investigation.

Consistent with a role for Bop1 in rRNA processing and ribosome maturation, Bop1 appears to be ubiquitously expressed irrespective of the tissue type (Fig. 2). Moreover, *bop1* mRNA levels begin to rise in mid to late G<sub>1</sub>, coincident with the timing of rRNA synthesis (24, 65, 70). As expected of a protein that plays a role in ribosome maturation, Bop1 appears

to be highly conserved throughout evolution. The mouse Bop1 displays >90% amino acid identity to its human ortholog (KIAA0124) and ~45% identity to a sequence in *Saccharomyces cerevisiae* (YMR049c). A BLAST search in the currently available databases also reveals protein coding sequences highly homologous to Bop1 in such diverse organisms as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*.

In this report, we show for the first time the effect of a nucleolar protein on rRNA processing in a mammalian system. Only a few other mammalian proteins have been implicated in rRNA processing to date, based on the function of their yeast homologs. For example, p120 was originally identified as a tumor proliferation antigen (33) and was later implicated in rRNA processing, since deletion of its yeast homolog, Nop2p, causes a block in the processing of the 27S pre-rRNA to the mature 25S rRNA (36). Consistent with a role in rRNA processing, p120 was shown to cofractionate with the 60-80S pre-ribosomal particles in HeLa cell extracts (30).

In yeast, several proteins whose depletion specifically affects processing of the 25S branch of rRNA processing have been identified. For example, depletion of the yeast Nop4 protein results in the inhibition of the 25S rRNA maturation as well as 60S ribosomal-subunit accumulation, while production of the 18S rRNA is unaffected (74). Other examples of similar phenotypes result from depletion of members of the DEAD box family of putative ATP-dependent RNA helicases including DRS1 (63), Dbp6p (41), Dbp7p (15), and Spb4p (18), as well as nucleolar proteins Nip7p (87) and Nop8p (86). These observations underscore the notion that processing of the 25S/28S branch of rRNA requires complexes of numerous proteins, whose nature is only beginning to be understood. In *Xenopus*,

depletion of the U8 snoRNA (56) also has effects similar to Bop1 $\Delta$  expression, namely, an incomplete inhibition of 36S pre-rRNA processing but a complete block of 32S pre-rRNA processing, leading to inhibition of 28S and 5.8S rRNA production. Immunoprecipitation of Bop1 followed by RNA blotting with an anti-U8 RNA probe failed to detect the U8 RNA (data not shown). In addition, immunoprecipitation of Bop1 from cells metabolically labeled with radioactive phosphate followed by PAGE also did not reveal the presence of small RNAs (data not shown). These results suggest that Bop1 is unlikely to be mediating the interaction of U8 or other snoRNAs with the rRNA precursor.

Processing of pre-rRNA and ribosome biogenesis appear to be highly coordinated (19, 49, 64, 80, 81). Consistent with this notion, there is a dramatic decrease in the amount of mature 60S ribosomal subunits when maturation of the 28S and 5.8S rRNAs is blocked as a result of Bop1 $\Delta$  expression (Fig. 9). The deficit in the 60S ribosomal subunit may be a direct result of the inability to produce its cognate mature rRNAs. However, we cannot rule out the possibility that Bop1 may also have the distinct function of coordinating 60S ribosomal assembly and that this function is compromised by Bop1 $\Delta$ .

Analysis of the primary sequence of Bop1 does not suggest an apparent enzymatic activity, although it does show features of a short-lived, regulatory protein that may participate in critical protein-protein interactions. The Bop1 sequence contains clusters of charged amino acid residues, known as PEST sequences, often associated with regulatory and short-lived proteins (13, 61, 67). Similar clusters are also observed in a number of nucleolar proteins (71). A putative nuclear localization signal in Bop1 is located at aa 360 to 366 (PRQRKMR; underlining indicates positively charged residues) (20, 25). Data from the present study with Bop1 $\Delta$  show that the N-terminal 231 aa are not needed for nucleolar location or complex formation with RNP particles but are necessary for critical functions illustrated by the consequences of Bop1 $\Delta$  expression.

Bop1 contains four WD40 repeats (Fig. 1), a sequence motif found in a large variety of regulatory proteins and known to mediate protein-protein interactions (26, 27, 51, 52, 72). WD40 proteins have been implicated in a number of cellular processes, including various stages of RNA metabolism (3, 6). At present, at least two other nucleolar proteins that contain WD40 repeats have been identified: the yeast SOF1 (38) and the related (but not orthologous) human protein hU3-55k (60). Inasmuch as a number of WD40 repeat proteins are known to form large multiprotein complexes, Bop1 may mediate protein-protein interactions that are important for the formation and activities of nucleolar RNPs. Future studies aimed at the identification and characterization of the proteins with which Bop1 interacts may yield new insights into the structural organization and mechanism of action of the nucleolar pre-rRNA-processing machinery.

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